Manganese superoxide dismutase and cardiovascular aging phenotypes in mice

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Dedication

I would like to dedicate this Master's work to my parents, grandparents, and all extended family that has provided unwavering support and encouragement throughout my education. The strong foundation of determination, perseverance, and strong work ethic laid by parents has guided me through exceptional opportunities and experiences, and I hope more are to come. Without their love and guidance I would not be where I am today and I strive to continue to make them proud.

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List of Abbreviations

Ach: acetylcholine

ANG II: angiotensin 2

ATP: adenosine triphosphate

BH_2: dihydrobiopterin

BH_4: tetrahydrobiopterin

BMP: bone morphogenetic protein

CAVD: calcific aortic valve disease

CDKN2A: cyclin-dependent kinase inhibitor 2A

cGMP: cyclic guanosine monophosphate

COX: cyclooxygenase

CuZnSOD: Copper Zinc superoxide dismutase

DJ-1: Parkinson protein 7

DNA: deoxyribonucleic acid

EC: endothelial cells

ecSOD: extra cellular superoxide dismutase

EDHF: endothelium-derived hyperpolarizing factor

eNOS: endothelial nitric oxide synthase

FAD: flavin adenine dinucleotide

FMN: flavin mononucleotide

FOXO: forkhead box, sub-group O

Gpx: glutathione peroxidase

GSH: glutathione

GSSG: glutathione disulfide

GTP: guanosine triphosphate

GTPCH-1: GTP-cyclohydrolase I

H_2O: water

H_2O_2: hydrogen peroxide

ICAM: intercellular adhesion molecule

IFN-γ: interferon-gamma

IL-1β: interleukin-1beta

iNOS: inducible nitric oxide synthase

LPS: lipopolysaccharide

LVIDd: left ventricular internal dimension diastole

LVIDs: left ventricular internal dimension systole

MMP2: metalloproteinase

MnSOD: Manganese superoxide dismutase

Msx2: msh homeobox 2

NAD(P)H oxidase: nicotinamide adenine dinucleotide phosphate-oxidase

NAD(P)H: nicotinamide adenine dinucleotide phosphate

NF-κB: nuclear factor-kappa B

nNOS: neuronal nitric oxide synthase

NO: nitric oxide

NOX: NAD(P)H oxidase

NOS: nitric oxide synthase

Nqo1: NAD(P)H dehydrogenase quinone 1

Nrf2: nuclear factor 2

O_2: molecular oxygen

$O_2 \cdot^-$: superoxide

$ONOO^-$: peroxynitrite

PCR: polymerase chain reaction

$PGF_{2\alpha}$: prostaglandin F2-alpha

PGI2: prostacyclin

Prx: peroxiredoxin

RI-RR: reactive oxygen species induced-reactive oxygen species-released

RNA: ribonucleic acid

ROS: reactive oxygen species

Runx2: runt-related transcription factor 2

sGC: soluble guanylate cyclase

SIRT: sirtuin

SNP: sodium nitroprusside

SOD: superoxide dismutase

Sp7: trans-acting transcription factor 7

TGF-β: tumor growth factor-beta

TNF-α: tumor necrosis factor-alpha

Trx: thioredoxin

VCAM: vascular cell adhesion molecule

VSMC: vascular smooth muscle cells

WRN: Werner gene

XDH: xanthine dehydrogenase

XO: xanthine oxidase

INTRODUCTION

Cardiovascular disease affects at least 1 in 3 Americans, and is the leading cause of death in the United States (Roger et al., 2011). There are many risk factors that contribute to cardiovascular disease, and a vast amount of research has focused on the mechanisms that have contributed to initiation and progression of the disease. Increasing age, a major risk factor, is associated with increases in reactive oxygen species (ROS), which is thought to contribute to the development of age-related cardiovascular disease. Previous work in aged humans and animals has shown that NAD(P)H oxidase (Nox) contributes to age-related endothelial dysfunction and vascular fibrosis, and that reducing superoxide dismutase 1 (SOD1, cytosolic), SOD2 (mitochondrial), or SOD3 (extracellular) worsens endothelial function with aging (Brown, Chu, Lund, Heistad, & Faraci, 2006; Brown, Didion, Andresen, & Faraci, 2007; Didion, Kinzenbaw, Schrader, & Faraci, 2006; Didion et al., 2002; Fukai, 2009; Lund, Chu, Miller, & Heistad, 2009). In vitro experiments also suggest that, once initiated, ROS production may self-perpetuate (ROS-induced ROS-release) and further accelerate development of endothelial dysfunction and disease (A. E. Dikalova, Bikineyeva et al., 2010).

There is mounting of evidence that suggests sirtuin deacetylases play a major role in protecting against a number of age-related diseases, and may protect against vasomotor dysfunction with aging. For example, SIRT6-deficient mice have profound reductions in bone mineral density, exaggerated

kypholordosis, and a dramatically shortened lifespan compared to their wild-type littermates (Mostoslavsky et al., 2006). Furthermore, the observation that SIRT1-dependent deacetylation of endothelial nitric oxide synthase is essential for eNOS activation provides a more direct role of sirtuins in the regulation of vasomotor function. How sirtuin expression levels change with aging in different cardiovascular tissues, however, remains largely unknown.

One of the most common consequences of aging is stiffening of the aorta and aortic valve secondary to calcification and fibrosis. We have previously shown that valvular calcification is strongly associated with increases in oxidative stress and reductions in antioxidant defense mechanisms in humans (Miller, Chu et al., 2008). Several studies have shown that the balance between oxidative stress and nitric oxide bioavailability is an important determinant of vascular and valvular calcification *in vitro* (Miller, Chu et al., 2008; Weiss, Ohashi, Miller, Young, & Heistad, 2006; Yoshii et al., 2006). Furthermore, expression and activity of a number of aging-related genes has been shown to be regulated by oxidative stress. We are, however, not aware of data experimentally determining this link *in vivo*.

Thus, the aims of the current study were as follows: 1) to determine whether reducing mitochondrial antioxidant, manganese superoxide dismutase (MnSOD), results in maladaptive changes in pro- and anti-oxidant gene expression due to ROS-induced ROS-release with aging, 2) to determine whether reducing mitochondrial antioxidant, MnSOD, exacerbates reductions in aging-associated gene expression, sirtuins, and 3) to examine the consequences

of reducing mitochondrial antioxidant, MnSOD, on aortic and aortic valve function. The overall aim of this work was to test two over-arching working hypotheses. First, that molecular changes associated with aging are markedly different between aorta and aortic valve. Second, that reducing mitochondrial antioxidant, MnSOD, would increase oxidative stress and reduce nitric oxide bioavailability, exaggerate reductions in longevity-related genes, and ultimately accelerate age-related aortic and aortic valve dysfunction.

The specific hypotheses we tested in the current work can be broken down into three categories: 1) changes in aorta with aging and/or MnSOD deficiency, 2) changes in aortic valve with aging and/or MnSOD deficiency, and 3) differences between aorta and aortic valve with aging and/or MnSOD deficiency.

Hypotheses

Changes in aorta with aging and/or MnSOD deficiency

Hypothesis 1: Overall antioxidant capacity will be reduced and pro-oxidant gene expression will be increased with aging in aorta.

Hypothesis 2: Reduction of MnSOD in aorta will increase oxidative stress and NAD(P)H oxidase isoform expression due to the initiation of ROS-induced ROS-release.

Hypothesis 3: Sirtuin expression will be significantly reduced in aorta with increasing age, and these reductions will be exacerbated in MnSOD-deficient mice.

Hypothesis 4: Increasing age will exacerbate endothelial dysfunction in MnSOD-deficient mice due to further amplification of ROS-induced ROS-release from age-related mitochondrial dysfunction and age-related increases in NAD(P)H oxidase expression .

Changes in aortic valve with aging and/or MnSOD deficiency

Hypothesis 5: Increasing age will significantly reduce overall antioxidant capacity and antioxidant transcription factor expression and an increase of oxidative stress in aortic valve.

Hypothesis 6: Reduction of MnSOD in aortic valve will increase NAD(P)H oxidase isoform expression due to the initiation of ROS-induced ROS-release.

Hypothesis 7: Sirtuin expression will be significantly reduced in aortic valve with increasing age, and losses of MnSOD will exaggerate these effects.

Hypothesis 8: Increasing age will exacerbate aortic valve dysfunction in MnSOD-deficient mice due to further amplification of ROS-induced ROS-release from age-related mitochondrial dysfunction and age-related increases in NAD(P)H oxidase expression in the valve.

Differences between aorta and aortic valve with aging and/or MnSOD deficiency

Hypothesis 9: Reductions in expression of antioxidants and antioxidant transcription factors with aging will be greater in aortic valve compared to aorta.

Hypothesis 10: Reductions of MnSOD will initiate ROS-induced ROS-release in both aorta and aortic valve, and these effects will be exacerbated by aging in both tissues.

Hypothesis 11: Reductions in sirtuin expression with increasing age will be greater in aortic valve compared to aorta.

LITERATURE REVIEW

Endothelial Function

Endothelial cells (EC) line the lumen of arteries as a monolayer of cells and play a major role in the regulation of cardiovascular homeostasis. Within the walls of the vessels there is a delicate balance between nitric oxide (NO) bioavailability and levels of reactive oxygen species (ROS). NO bioavailability is largely determined by the production of NO via nitric oxide synthase (NOS). Furchgott and Zawadzki are credited with the discovery of NO and its role as a signaling molecule between endothelial cells and vascular smooth cells acting as primarily as an endothelium-derived relaxing factor (Furchgott, Carvalho, Khan, & Matsunaga, 1987). Briefly, NO produced in EC is then transported to the neighboring smooth muscle cells, where NO interacts with soluble guanylate cyclase (sGC) and guanosine triphosphate (GTP) to produce cyclic guanosine monophosphate (cGMP). Ultimately, cGMP will signal vasorelaxation in smooth muscle cells (Thomas, Witting, & Drummond, 2008). This process will continue as long as there is ample amount of NO bioavailability in the vasculature.

Reactive oxygen species (ROS) are natural cellular byproducts and two main sources of ROS are NAD(P)H oxidases and mitochondria (Finkel & Holbrook, 2000). The balance between NO bioavailability and ROS is preserved by antioxidant defense systems (i.e. catalase, superoxide dismutases), however, several investigators have suggested that the major driver for an increased risk of age-related diseases is an increase in reactive oxygen species (ROS) (Csiszar et al., 2002; Forstermann, 2010; Hamilton, Brosnan, Al-Benna, Berg, &

Dominiczak, 2002; Lakatta & Schulman, 2004; Schulz, Jansen, Wenzel, Daiber, & Munzel, 2008; Wilcox et al., 1997). ROS are efficient at inactivating nitric oxide (NO), which can subsequently lead to further production of ROS (Forstermann, 2010). The term "oxidative stress" is defined as an increase production of ROS which exceeds the antioxidant defense systems and contributes to cellular toxicity (Drummond, Selemidis, Griendling, & Sobey, 2011; Forstermann, 2010). Oxidative stress, collectively, inhibits NO production and decreases NO bioavailability leading to various age-related cardiovascular diseases.

Nitric Oxide Synthases

Nitric oxide synthase function is the conversion of L-arginine to L-citrulline and NO in order to promote endothelium-dependent relaxation. The structure of NOS is a homodimer which is comprised of a reductase domain (C-terminal) and oxygenase domain (N-terminal). The reductase domain is the binding site of NADPH, FAD, and FMN. Tetrahydrobiopterin (BH_4), heme, L-arginine, and zinc ion bind to the oxygenase domain. In the presence of intracellular calcium (Ca^{2+}), calmodulin also binds between the two domains (Andrew & Mayer, 1999; Forstermann, 2010).

There are three distinct NOS isoforms each possessing unique responsibilities in the body. Endothelial NOS (eNOS) and neuronal (nNOS) are classified as Ca^{2+}-dependent, whereas, inducible NOS (iNOS) activity is Ca^{2+}-independent and activated by cytokines and other inflammatory markers (Andrew & Mayer, 1999). However, eNOS activity has also been shown to be induced in

Ca^{2+}-independent environments, particularly following the phosphorylation of residues on the eNOS structure (Forstermann, 2010). Location of the isoforms in the body is also specific to each isoform. For instance, nNOS is largely expressed in nervous tissue and also skeletal muscle (Andrew & Mayer, 1999). eNOS expressed in endothelial cells is highly involved in the generation of NO-dependent relaxation in blood vessels (Andrew & Mayer, 1999). Similarly to that of eNOS, iNOS plays a critical role in the cardiovascular system especially in disease states. Induced by inflammatory mediators, iNOS impairs NO production by eNOS via its own high output of NO (Kessler, Bauersachs, Busse, & Schini-Kerth, 1997). Overall, each isoform presents unique expression and activity in the body.

NO biosynthesis begins at the reductase domain where NADPH donates electrons. Calmodulin binds to NOS due to calcium-induced binding to NOS (Forstermann, 2010). Calmodulin modulates the flow of electrons and the initiation of electron transfer from the C-terminal to N-terminal. Electrons flow to the heme center. Important NOS cofactors L-arginine and BH_4 are bound to the oxygenase domain. The zinc ion is also important cofactor for NOS because it has been found that it may help with the stereospecificity of BH_4 binding (Andrew & Mayer, 1999). L-arginine is then hydroxylated from L-arginine to N^G-hydroxy-L-arginine and is quickly oxidized to L-citrulline and NO. Largely, NO biosynthesis is increased with the presence of Ca^{2+} because of the increase binding and flow of electrons by calmodulin. However, in some states such as increased shear

stress of blood flow, estrogens, insulin, or bradykinin, has been found to initiate NO production when there are no changes in Ca^{2+} levels (Forstermann, 2010). This may be explained by the phosphorylation of multiple sites within NOS that induces the increase of flow of electrons from the reductase domain to the oxygenase domain (Andrew & Mayer, 1999; Forstermann, 2010). Collectively, production of NO can be mediated by either Ca^{2+} dependent or Ca^{2+} independent mechanisms.

Essential NOS cofactors

Endothelial function is highly dependent on proper NO production from NOS and in turn NOS is highly dependent on the presence of the cofactors, BH_4 and L-arginine. BH_4 and L-arginine are the cofactors that are essential to the production of NO. However, disruption of cofactor bioavailability can significantly impair NO production leading to decreased NO bioavailability and ultimately endothelial dysfunction.

BH_4 bioavailability is regulated by multiple factors such as positive regulation by GTP-cyclohydrolase I (GTPCH I) and negative regulation by ROS. As an essential cofactor, BH_4 promotes L-arginine binding to the NOS heme iron binding site (Touyz & Briones, 2011). Binding of BH_4 to NOS also contributes to stabilization of the NOS dimer formation (Touyz & Briones, 2011). However, BH_4 is easily oxidized to its inactive form BH_2 by reactive oxygen species, which can lead to reduced bioavailability of BH_4. In disease states where reactive oxygen species levels are high, BH_4 is readily oxidized to $BH_3\cdot$ by peroxynitrite ($ONOO^-$),

before further oxidation to BH_2. $ONOO^-$ is produced by the interaction between superoxide ($O_2\cdot^-$) and NO (Forstermann, 2010). Increased levels of $ONOO^-$ significantly reduces BH_4 levels therefore, leading to impaired NOS function.

Attenuation of BH_4 levels can also lead to the "uncoupling" of NOS. Uncoupled NOS is unable to produce NO, but rather generates $O_2\cdot^-$ and furthering ROS production (Forstermann, 2010; Landmesser et al., 2003). Collectively, the presence of BH_4 is an essential component to the production of NO and ultimately endothelial function.

L-arginine is endogenously inhibited by asymmetric dimethyl-L-arginine (ADMA), blocking L-arginine binding to NOS. However, this inhibition is tightly regulated by the presence of dimethylaminohydrolase (DDAH), which is responsible for the degradation of ADMA. ADMA and DDAH are redox sensitive, and in the presence of oxidative stress DDAH levels are reduced and ADMA levels are increased (Goumas, Tentolouris, Tousoulis, Stefanadis, & Toutouzas, 2001). This imbalance between ADMA and L-arginine functionally creates an L-arginine deficient environment, resulting in NOS dysfunction (Forstermann, 2010; Goumas et al., 2001).

Hydrogen Peroxide as an Endothelium-Derived Hyperpolarizing Factor

Hydrogen Peroxide (H_2O_2) has been implicated to be an endothelium-derived hyperpolarizing factor (EDHF) (Shimokawa & Matoba, 2004). In the presence of superoxide, NO bioavailability is reduced and NO-dependent endothelial function is impaired (Forstermann, 2010). Superoxide is degraded

either spontaneously or by superoxide dismutases (SODs) to H_2O_2. In various studies H_2O_2 can alternatively induce vascular relaxation (Cherry, Omar, Farrell, Stuart, & Wolin, 1990; Iesaki, Gupte, Kaminski, & Wolin, 1999; Sobey, Heistad, & Faraci, 1997). It is believed that H_2O_2 originates from the endothelial cell and diffuses to the vascular smooth muscle cell where it activates K^+ channels to hyperpolarize the vessel (Edwards, Dora, Gardener, Garland, & Weston, 1998; Edwards et al., 2000; Matoba et al., 2000). The production of superoxide by eNOS may be a significant source of the H_2O_2 contributing to H_2O_2-derived EDHF, as Matoba et al have previously shown that eNOS knockout mice significantly had reduced H_2O_2-derived EDHF responses (Shimokawa & Matoba, 2004). Collectively, increases in H_2O_2 production may be an important compensatory mechanism under conditions of reduced NO bioavailability.

Prostaglandins

Prostaglandins also contribute to endothelium derived relaxing factors, specifically prostacyclin (PGI2). Production of PGI2 is mediated by cyclooxygenase (Wilcox et al.) (Wilcox et al.) enzyme. COX metabolizes arachidonic acid to produce PGI2 (Rosolowsky & Campbell, 1993). PGI2 then travels to the vascular smooth muscle where it acts on cyclic-AMP in the vascular smooth muscle to induce relaxation (Shimokawa & Matoba, 2004). It has been shown that the inhibition of COX via indomethacin administration significantly impairs endothelial relaxation, suggesting that the formation of PGI2 via COX is critical to endothelium-dependent relaxation (Rosolowsky & Campbell, 1993).

Sources of ROS

ROS is produced by a variety of sources in the body, however, there are four main producers that predominate the ROS production in the vascular walls: NAD(P)H oxidase, mitochondrial respiration, xanthine oxidase, and uncoupled eNOS (A. E. Dikalova, Bikineyeva et al., 2010; Thomas et al., 2008; Touyz & Briones, 2011). It has been shown that these ROS producing enzymes may act on one another to initiate a feed-forward phenomenon that will potentiate oxidative stress and impairment of endothelial function (S. Dikalov, 2011; A. E. Dikalova, Bikineyeva et al., 2010; Landmesser et al., 2003).

NAD(P)H Oxidases

Nicotinamide adenine dinucleotide phosphate (NAD(P)H)-oxidase (Nox) family of enzyme complexes is comprised of seven different isoforms (Drummond et al., 2011; Forstermann, 2010; Thomas et al., 2008). Nox are enzymes that are found in cells such as endothelial cells and vascular smooth muscle cells. However, Nox is not limited to endothelial and vascular smooth muscle cells, but also play a major role in the immune defenses such as phagocytosis (Drummond et al., 2011). In general, the main function of Nox enzymes is to produce ROS (Drummond et al., 2011), and currently is the only known family of enzymes to have ROS production as its only function. Depending on the Nox isoform, superoxide ($O_2\cdot$) or hydrogen peroxide (H_2O_2) is the major product from Nox. In the vasculature, four of the seven Nox isoforms are present: Nox1, Nox2, Nox4, and Nox5 (Drummond et al., 2011).

Furthermore, Nox1, Nox4, and Nox5 are present in vascular smooth muscle cells, but Nox2 is not present (Drummond et al., 2011; Thomas et al., 2008). It has been found that each Nox isoform in vasculature plays unique role in the development of impaired endothelial function.

Nox1 is found in both endothelial cells and vascular smooth muscle cells, however, its role in vascular smooth muscle cells (VSMC) biology is well understood compared to its role in endothelial cell biology (Drummond et al., 2011). Atherosclerotic animal models have provided a vast amount of knowledge about Nox1 and its role in vascular remodeling, increased ROS production, neointimal formation (Bengtsson, Gulluyan, Dusting, & Drummond, 2003; Niu et al., 2010; Sheehan et al., 2011). Production of superoxide by Nox1 is found to be upregulated and contribute to hypertension in animal models (Akasaki et al., 2006; S. I. Dikalov et al., 2008; A. Dikalova et al., 2005). Collectively, Nox1 is found largely in the vascular smooth muscle and potentiates ROS production in hypertension and atherosclerotic lesions.

Nox4 research has a rather controversial background and is hard to determine its exact role in VSMC. Similar to other Nox isoforms, the majority of the evidence suggests that it plays a detrimental role in the vasculature. Pedruzzi et al has demonstrated that Nox4-derived H_2O_2 induces oxidative stress in aortic vascular smooth muscle cells (Pedruzzi et al., 2004). They also showed that this oxidative stress leads to cell death and contributes to the pathogenesis of atherosclerosis (Pedruzzi et al., 2004). However, some work has suggested

that Nox4 may have a protective role in the vasculature, such as promoting endothelial cell survival (Basuroy, Tcheranova, Bhattacharya, Leffler, & Parfenova, 2010; Datla et al., 2007). The conflicting data regarding Nox4 and its potential opposing role in different cell types makes it difficult to fully understand the net effect of Nox4 activation in the vasculature.

Nox2 only found in EC and not in VSMC has been characterized as the mediator of endothelial injury. Composed of necessary subunits p47phox and p22phox, Nox2 expression is increased in the presence of pro-inflammatory stimuli (S. Dikalov, 2011; Drummond et al., 2011). It has been well established, in angiotensin II induced hypertension, is the causation of the upregulation of Nox2 and also Nox1 in vasculature (S. Dikalov, 2011; Drummond et al., 2011). Numerous investigators have improved vascular function by administering apocynin, an NAD(P)H oxidase inhibitor (Bendall et al., 2007; Rey, Cifuentes, Kiarash, Quinn, & Pagano, 2001). However, Rey et al was the first group to demonstrate that specifically inhibiting Nox2, gp91 ds-tat peptide, attenuates vascular oxidative stress and reduces systolic blood pressure in hypertensive mouse model (Rey et al., 2001). It has also been well established in aging models that inhibition of Nox2 improves endothelial function (Csiszar et al., 2002; Donato et al., 2007; Hamilton et al., 2002); providing evidence that Nox2 mediates age-induced endothelial dysfunction. The fact that inhibition of Nox2 improves endothelial function strongly suggests that Nox2 plays a pivotal role in the pathophysiology of multiple vascular diseases.

Mitochondria

The mitochondria are often considered as the “energy house” of the cell. Largely, mitochondria are known for its production of adenosine-5’-triphosphate (ATP), however, the byproduct—ROS—is often given little attention. This is largely due to the fact that endothelial cells have a low metabolic rate (S. Dikalov, 2011; Y. Zhang et al., 2009). It has been well established, recently, that mitochondria is one of the main sources of ROS in the vasculature (S. Dikalov, 2011; D. X. Zhang & Gutterman, 2007). Consequently, the paradigm has shifted from NAD(P)H oxidase being the sole driver of ROS in endothelium, to examining the effects of mitochondria-derived ROS in the vasculature (S. Dikalov, 2011).

The electron transport chain is the origin of ROS in the mitochondria, where the four main complexes transfer electrons to assemble ATP (S. Dikalov, 2011; D. X. Zhang & Gutterman, 2007). Briefly, complex I transfers electrons from nictotinamide adenine dinucleotide (NADH) to complex II. Ubiquinone mediates the flow of electrons to complex III. Finally, electrons pass through complex IV to form H_2O and then pumps H+ into the inter membrane (S. Dikalov, 2011). The respiration process of the mitochondria is the known source of ROS. Predominantly, complex I and complex III have been recognized as primary sources of ROS in the electron transport chain (D. X. Zhang & Gutterman, 2007). These two complexes “leak” superoxide ($O_2{\cdot}^-$) on the matrix side of the mitochondria. It has been recently shown that complex II may also form ROS via

a process called reverse electron transport, however this is not completely understood (Ackrell, Kearney, & Mayr, 1974; Lambert, Buckingham, Boysen, & Brand, 2008). Once superoxide is produced in the mitochondria it is not easily diffused across in the cell membrane, leaving the mitochondria very susceptible to DNA damage, signaling of cell death (apoptosis), and other ROS-induced effects (D. X. Zhang & Gutterman, 2007). In order to reduce the accumulation of ROS the mitochondria has a "built in" antioxidant defense system.

Manganese superoxide dismutase (MnSOD), localized only in the mitochondrial matrix, and scavenges $O_2\cdot^-$ and reduces it to H_2O_2. Once $O_2\cdot^-$ is converted to H_2O_2 by MnSOD it can readily diffuse across the cell membrane (D. X. Zhang & Gutterman, 2007). However, increased mitochondrial ROS production as is the case in the presence of mitochondrial diseases or aging, can lead to oxidative stress in the mitochondria and impairment of ATP production and other adverse effects (Ballinger, 2005). Recently, it has also been shown mitochondrial oxidative stress can act upon or signal other external ROS production, such as NAD(P)H oxidase (S. Dikalov, 2011; A. E. Dikalova, Bikineyeva et al., 2010).

Xanthine Oxidase

Xanthine oxidase is derived from irreversible proteolysis of xanthine dehydrogenase (XDH) (Forstermann, 2010; McNally, Saxena, Cai, Dikalov, & Harrison, 2005). Xanthine oxidase (XO) reduces xanthine and hypoxanthine substrates to produce $O_2\cdot^-$ and H_2O_2 (McNally et al., 2005). Studies have shown

that inhibition of XO improves endothelial function in patients (Ohara, Peterson, & Harrison, 1993). However, McNally et al showed that production of ROS by XO is highly redox-sensitive, and specifically dependent of NAD(P)H oxidase expression (McNally et al., 2005).

Uncoupled eNOS

eNOS can be in two functional forms, coupled and uncoupled. Coupled eNOS produces the protective NO, whereas uncoupled eNOS manufactures $O_2\cdot^-$ that promotes deleterious effects on vasculature. The main determinant of coupled eNOS is BH_4 bioavailability. High levels of BH_4 contribute to the stabilization of dimer structure and the coupled state of eNOS to synthesize NO (Montezano & Touyz, 2011). However, a reduction of BH_4 bioavailability influenced by oxidative stress is due to the increase in $ONOO^-$ formation, reductions of GTPCH expression, and oxidation to the inactive form, BH_2. The homodimer structure is affected by reductions of BH_4, which decreases the stabilization of the dimer and prevents L-arginine to bind to the oxygenase domain (Moens & Kass, 2006; Montezano & Touyz, 2011). Inability for L-arginine to bind, electrons that are transferred through the haem are now donated to molecular O_2 to produce $O_2\cdot^-$ (Moens & Kass, 2006; Montezano & Touyz, 2011). Change in the dimer structure prevents L-arginine binding to eNOS causing electrons to be donated to molecular O_2 with an end product of $O_2\cdot^-$ (Montezano & Touyz, 2011).

Antioxidant Defenses

Build up ROS in the cell can contribute to numerous cellular deleterious changes such as, DNA damage, cell growth, and cell apoptosis (Finkel & Holbrook, 2000; Podlutsky, Ballabh, & Csiszar, 2010; Schiffrin, 2008; Touyz & Briones, 2011). Through the course of evolution the body has developed an antioxidant defense system to break down ROS into reduced forms and promote vascular homeostasis. Superoxide dismutases and hydrogen peroxide-degrading enzymes are responsible for this, for instance, superoxide dismutases break down $O_2^{\cdot -}$ into H_2O_2 and O_2, and hydrogen peroxide-degrading enzymes reduce H_2O_2 into H_2O. However, each enzyme has its own unique key differences that contribute to vascular homeostasis and to preserve NO bioavailability.

SOD's

There are three different isoforms of superoxide dismutases (SOD): copper-zinc SOD (CuZnSOD), located in the cytoplasm; extracellular SOD (ecSOD), present in the extracellular matrix; and manganese SOD (MnSOD), found strictly in the mitochondria (Forstermann, 2010). Genetically reducing either CuZnSOD, ecSOD, or MnSOD in animals has found to increase oxidative stress and decrease NO bioavailability in vasculature (Didion et al., 2006; Didion et al., 2002; Gongora et al., 2006; Jung et al., 2003; Y. Zhang et al., 2009). It is also important to note that reducing one SOD isoform does not cause a compensatory increase in the other SOD isoform expressions, and that

overexpressing any one SOD isoform may stave off pathophysiologies associated with aging, but does not extend lifespan (Faraci & Didion, 2004; Perez et al., 2009). Furthermore, of the three SOD isoforms, MnSOD is essential to life. Mitochondria is a major source of ROS, consuming nearly 5% of all molecular O_2 and converting it to $O_2{\cdot}^-$ due to the electron transport chain (Faraci & Didion, 2004). Therefore, the mitochondria are highly susceptible to oxidative damage and MnSOD is the first line of defense to reduce ROS accumulation. Li et al have demonstrated the importance of MnSOD is to life by globally knocking out MnSOD. It was found that these mice die shortly after birth due to the development of dilated cardio myopathy (Y. Li et al., 1995).

Transcription factors and external/internal stimuli are responsible for the regulation of SOD isoforms expression levels. Transcription factor protein, NF-κB, an important protein in inflammation and immunity defenses, promotes cell survival in the presence of ROS (Morgan & Liu, 2010). NF-κB has been found to induce and mediate MnSOD and CuZnSOD expression (Dhar, Tangpong, Chaiswing, Oberley, & St Clair, 2011; Rojo, Salinas, Martin, Perona, & Cuadrado, 2004; Zelko, Mariani, & Folz, 2002). MnSOD and CuZnSOD also share common transcription factors specificity protein-1 (Sp1) and activator protein-1 (AP1) (M. Li, Chiu, Mossman, & Fukagawa, 2006; Zelko et al., 2002). MnSOD is also positively regulated by transcription factor Forkhead box class O-3a (FOXO3a) (M. Li et al., 2006). ecSOD shares very similar genomic structure to that of CuZnSOD, however, ecSOD transcription factors are different than that of

CuZnSOD and MnSOD (Zelko et al., 2002). ecSOD has sites for Kruppel-like and Ets-family transcription factors, but little research has focused on the functional importance of these ecSOD transcription factors (Zelko et al., 2002).

Transcription factor activation and binding are promoted by numerous stimuli in and outside the cell. CuZnSOD is highly induced by noxious stimuli such as hydrogen peroxide and induction of oxidative stress by paraquat treatments (Yoo, Chang, & Rho, 1999; Zelko et al., 2002). Instead MnSOD and ecSOD expression levels are regulated by pro-inflammatory cytokine stimuli. Tumor necrosis factor-alpha (TNF-α), interleukin-1beta (IL-1β), and interferon-γ (IFN-γ) have been shown to increase MnSOD expression in vascular smooth muscle cells (VSMC) (Stralin & Marklund, 2000; Zelko et al., 2002). IFN-γ has also been shown to upregulate ecSOD VSMC, however, expression is down regulated in the presence of TNF-α (Stralin & Marklund, 2000; Zelko et al., 2002). Collectively, stimulation by external/internal factors initiates the activity of transcription factors to promote the antioxidant defenses.

Hydrogen peroxide-degrading enzymes

Hydrogen peroxide (H_2O_2) is not a charged molecule like superoxide, however, it is still considered to be a reactive oxygen species. Its classification as a ROS is largely due to the fact H_2O_2 is easily converted to a hydroxyl group ($OH^{\cdot}$) via the Fenton reaction (Nordberg & Arner, 2001). $OH^{\cdot}$ accumulation leads to lipid peroxidation and DNA/protein damage (Nordberg & Arner, 2001). Therefore, there are three main enzymatic proteins (catalase, glutathione

peroxidase, and peroxiredoxins) that degrade H_2O_2 to H_2O and O_2 to reduce the likelihood of the conversion to the highly toxic $OH^{\cdot}$.

Catalase

Catalase, a heme-containing homotetrameric protein, is one of the main hydrogen peroxide-degrading enzymes. Catalase is primarily localized in peroxisomes, however, catalase has been found in mitochondria of cardiomyocytes (Ho, Xiong, Ma, Spector, & Ho, 2004; Radi et al., 1991). Two major reactions constitute catalase activity: catalytic mode and peroxidatic mode (Chance, Sies, & Boveris, 1979; Ho et al., 2004). At high levels of concentration H_2O_2, a molecule of H_2O_2 reacts with the heme group of catalase to form compound I. Compound I then interacts with a second molecule of H_2O_2 to yield H_2O and O_2 (Chance et al., 1979; Ho et al., 2004), therefore, it is necessary to have two molecules of H_2O_2 to degrade H_2O_2. However, at low concentrations of H_2O_2 compound I can oxidize alcohols via the peroxidation reaction. This suggests that the peroxidation activity is favorable at low H_2O_2 concentration (Boveris, Oshino, & Chance, 1972; Keilin & Hartree, 1949). Catalase activity is not only regulated by H_2O_2 concentration, but also various exogenous and endogenous stimuli both positive and negative. For instance, increase in Nox4 via TGF-β signaling has been shown to down regulate catalase activity (Michaeloudes, Sukkar, Khorasani, Bhavsar, & Chung, 2011), whereas, Foxo3a stimulates catalase transcription (Bonekamp, Volkl, Fahimi, & Schrader, 2009).

Glutathione Peroxidase

Hydrogen peroxide-degrading enzyme, glutathione peroxidase (Gpx), is believed to reduce over 70% of all H_2O_2 produced in the cell due to higher affinity to H_2O_2 compared to catalase (Stanczyk, Gromadzinska, & Wasowicz, 2005). The main source of production of Gpx is in the liver, however, Gpx is produced in all cells (Hammond, Lee, & Ballatori, 2001; Stanczyk et al., 2005). Localized to the cytosol of the cell, but also located in the mitochondria (Rhee, Chae, & Kim, 2005; Stanczyk et al., 2005), it is largely responsible for the degradation of H_2O_2 and has been found to play a crucial role in the regulation of apoptosis and inhibition of lipid peroxidation (Hammond et al., 2001; Owen & Butterfield, 2010; Stanczyk et al., 2005). In order to reduce H_2O_2 to H_2O and O_2, Gpx oxidizes glutathione (GSH) to glutathione disulfide (GSSG). GSSG is quickly reduced back to GSH by the enzyme glutathione reductase at the expense of NADPH. GSH/GSSG ratio is often indicative of oxidative stress and overall cell health. In healthy cells, reduced GSH constitutes more than 90% of the cell GSH present, whereas GSSG makes up less than 10%. However, when oxidative stress is increased this ratio is reduced (Hammond et al., 2001; Owen & Butterfield, 2010; Stanczyk et al., 2005).

Peroxiredoxin

Peroxiredoxin (Prx), originally termed thioredoxin peroxidase, is the most recently discovered protein able to reduce H_2O_2 along with organic hydrogen peroxides and peroxynitrite (Immenschuh & Baumgart-Vogt, 2005; Nordberg & Arner, 2001). In brief, Prx's reactive cysteine residues catalyze the degradation

of H_2O_2 by oxidizing thioredoxin (Trx) as the electron donor to form H_2O and O_2 (Zhu, Santo, & Li, 2012). The oxidized Trx is then reduced back by thioredoxin reductase (Zhu et al., 2012). Six known isoforms comprise the Prx family: Prx I, Prx II, Prx III, Prx IV, Prx V, and Prx VI. Prx I is the most widely distributed in tissue and also at the cellular level. Prx V is the second most widely distributed Prx, and is found in cytoplasm, mitochondria, and peroxisomes. Prx II is localized in the nucleus and cytoplasm, and Prx IV in the extracellular space and lysosomes. Prx III and Prx VI are the only Prx isoforms that have been found to be specifically localized to one area—mitochondria and cytoplasm, respectively (Immenschuh & Baumgart-Vogt, 2005). Prx gene expression, explicitly Prx I, is induced by lipopolysaccharide (LPS) and heavy metals in liver tissue macrophages and cultured hepatic cells, respectively (Immenschuh & Baumgart-Vogt, 2005). Transcriptionally, Prx gene expression is regulated by Nrf2, a key gene regulator in oxidative stress environments (Immenschuh & Baumgart-Vogt, 2005; Zhu et al., 2012).

ROS-Induced ROS-Release Phenomenon

It has been found by many investigators that cytoplasmic ROS production can activate mitochondrial-derived ROS, vice versa, and may begin a vicious cycle contributing to ROS accumulation in the cell (A. E. Dikalova, Bikineyeva et al., 2010; Kimura et al., 2005). Dikalova et al has investigated this ROS-induced ROS-release (RI-RR) phenomenon in an angiontensin (Ang) II model. Ang II has been shown to upregulate Nox2 (Mehta & Griendling, 2007; Mollnau et al., 2002;

Seshiah et al., 2002; Touyz & Briones, 2011) in rats and mice, therefore, this increase in ROS production will lower NO bioavailability. Research has shown that Nox2-dervied ROS activates mitoK_{ATP}, a regulated potassium channel of the mitochondria. MitoK_{ATP} activation causes a rapid influx of K^+ and signals an enhancement of mitochondrial ROS production (S. Dikalov, 2011; Krenz et al., 2002). This finding suggests that there is a crosstalk occurring between NAD(P)H oxidases and mitochondria which perpetuate ROS production (see Figure 1). Dikalova et al (2010) has further demonstrated this crosstalk between these two ROS generating systems using both *in vivo* and *in vitro* models. It was shown that Ang II treated cells increased NAD(P)H oxidase activity, (consistent with previous findings of upregulation of NAD(P)H oxidase due to Ang II (Mehta & Griendling, 2007; Mollnau et al., 2002; Seshiah et al., 2002; Touyz & Briones, 2011)), and NAD(P)H oxidase activity was augmented even further in cells with MnSOD deficiency. However, cellular levels of ROS were nearly abrogated with treatment of mitoTEMPO, a specific mitochondrial superoxide scavenger. Collectively, this finding provides evidence that mitochondria-derived and NAD(P)H oxidase-derived ROS augment one another through a feed forward mechanism. This group also observed impaired aortic endothelial relaxation to acetylcholine in C57 mice treated with Ang II, however, Ang II treated vessels incubated with mitoTEMPO observed improved relaxation to basal levels (A. E. Dikalova, Bikineyeva et al., 2010). Largely, the body of evidence to support this RI-RR phenomenon has been performed in *in vitro,* and we are only beginning to

understand the role of RI-RR *in vivo*. **Currently, there is lack of investigation examining the effects RI-RR has on endothelial function and aortic valve function in unstressed conditions, and whether or not RI-RR is exacerbated with increasing age.**

Key Interactions between NO and ROS

Accumulation of ROS has significant negative effects on NO bioavailability which ultimately leads to endothelial dysfunction. Production of superoxide has been found to have deleterious implications on overall endothelial health, such as the formation of peroxynitrite ($ONOO^-$). $ONOO^-$ forms almost instant when NO and $O_2\cdot^-$ are just a few microns from each other (Pacher, Beckman, & Liaudet, 2007) and does not need an enzyme in order for this reaction to happen. Modest increases of NO and $O_2\cdot^-$ production will cause an exponential increase of $ONOO^-$ formation (Pacher et al., 2007), which will perpetuate the imbalance between ROS and NO. Accumulation of ROS impairs NO bioavailability by interfering the ability for DDAH to break down ADMA. The inability to reduce ADMA levels creates a functional blockade of the essential NOS cofactor, L-arginine, therefore leading to a decrease of NO bioavailability. $ONOO^-$ also oxidizes BH_4 to its inactive form BH_3. This reduction of BH4 bioavailability initiates the uncoupling of eNOS and augments the production of ROS further. Overall, the mechanisms whereby ROS interfere with NO bioavailability are multifactorial.

$ONOO^-$ has been shown to modify proteins that are essential to normal vasomotor function and likely to hinder the function of endothelial cells (Haddad et al., 1994; van der Loo et al., 2000). For instance, prostacyclin, important vasodilator, has been found its activity to be inhibited by $ONOO^-$ via the nitration of its tyrosine residues (Zou, Martin, & Ullrich, 1997). Nitrosylation induced by $ONOO^-$ also impacts antioxidant proteins, specifically MnSOD (MacMillan-Crow, Crow, & Thompson, 1998; van der Loo et al., 2000). MnSOD becomes inactivated after nitration of its tyrosine residues, which also contributes to an increase in ROS accumulation (MacMillan-Crow et al., 1998; van der Loo et al., 2000). Collectively, ROS can render vascular function by either degradation or nitrosylation of proteins.

Aging

Aging is one of the major risk factors for many degenerative diseases, especially cardiovascular disease. A large amount of research investigates the physiological changes that occur with increasing age. A major determinant of age-related diseases is the excess accumulation of ROS which promotes endothelial dysfunction. Our understanding of why aging increases ROS production and promotion of endothelial dysfunction remains rudimentary.

Reduction of Endothelial Function with Aging

Numerous studies have examined endothelial function with aging, and all demonstrated with increasing age there is significant impairment in endothelium-dependent relaxation (Csiszar et al., 2002; Donato et al., 2007; Hamilton et al.,

2002). There are multiple mechanisms that contribute to this observed decline in vascular function.

First, with increasing age there is strong evidence that supports accumulation of ROS and reduction of NO bioavailability (Blackwell et al., 2004; Brandes, Fleming, & Busse, 2005; Brown et al., 2007; Csiszar et al., 2002; Didion et al., 2006; Lund et al., 2009). As ROS increase, therefore, NO levels decrease, which contributes to vascular dysfunction. NAD(P)H oxidase is the main source for age-related production of ROS (Adler et al., 2003; van der Loo et al., 2000). There is also age-related decline of eNOS and L-arginine levels exacerbating ROS production (Berkowitz et al., 2003; Pacher et al., 2007). Antioxidant defenses are also found to decrease with age (Azhar, Cao, & Reaven, 1995). This is contributed by the formation of $ONOO^-$ which is found to inactivate MnSOD (MacMillan-Crow et al., 1998; Yamakura & Kawasaki, 2010) and reduction of Nrf2 expression that induces antioxidant defenses (Ungvari et al., 2011). Therefore the antioxidant defense system is overwhelmed and acts as a permissive mechanism to increase oxidative stress (Pacher et al., 2007; Sun et al., 2004).

Oxidative stress in aged vessels also contributes to induction of pro-inflammatory cytokines via nuclear factor-kappa B (NF-κB) activation (Csiszar, Ungvari, Koller, Edwards, & Kaley, 2003; Pacher et al., 2007; Ungvari, Kaley, de Cabo, Sonntag, & Csiszar, 2010). These include tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), iNOS, and vascular cytokine adhesion molecule (VCAM)

(Ungvari et al., 2010). This can lead to various changes of the vessel wall such as age-related arterial stiffness. Arterial stiffness is the result of increased fibrosis, matrix cross-linking, and thickening vessel walls and can be driven by transforming growth factor-beta (TGFβ), intercellular adhesion molecule-1 (ICAM), fibronectin, and metalloproteinase-2 (MMP2) (Lakatta, 2003; Lakatta & Schulman, 2004; Lakatta, Wang, & Najjar, 2009).

Reduction of Aortic Valve Function with Aging

The aortic valve is comprised of three cusps. Blood is pumped systemically by the left ventricle where it passes through the aortic valve to the rest of the body. In the healthy population, these leaflets create a tight seal during diastole to prevent backflow—regurgitation—into the left ventricle. Similar to the vasculature, however, aortic valves are highly susceptible to age-related stiffening and dysfunction.

Calcific aortic valve disease (CAVD) is the most common type of valvular disease that affects 5.2 million people, however, severe stenosis affects 3-4% of the population over the age of 65 (Beckmann, Grau, Sainger, Poggio, & Ferrari, 2010; Freeman & Otto, 2005). It is characterized as initial leaflet thickening to advance stages of calcium deposition causing obstruction of leaflet motion (Freeman & Otto, 2005). As the disease progresses from sclerotic valve to stenotic valve a decrease of cusp separation and increase of transvalvular velocity across the valve during systolic pressure is observed (Weiss et al., 2006). The major risk factor of CAVD is increasing age (Miller et al., 2010;

Rosenhek et al., 2004; Stewart et al., 1997; Weiss et al., 2006). Currently, the only treatment for CAVD is valve replacement. Pharmacological interventions have not effectively slowed the progression of the disease (Rosenhek et al., 2004).

CAVD leaves calcium deposits surrounding the annulus of the valve in early stages of the disease, and advances to the aortic side of the leaflet as the disease progresses (Sucosky, Balachandran, Elhammali, Jo, & Yoganathan, 2009). It is believed that CAVD begins with pro-osteogenic changes that cause valve interstitial cells to redifferentiate into osteoblast-like cells (Rajamannan et al., 2003). Bone morphogenetic protein (BMP) signaling is found to be the main osteogenic signaling cascade that contributes to the active calcification process in CAVD (Johnson, Leopold, & Loscalzo, 2006). BMPs are part of the TGF-β family and there are several BMP isoforms (Johnson et al., 2006). BMP2 and BMP4 have been found to be the regulators of bone mineralization in cardiovascular tissue and been found consistently to be upregulated in calcified cardiac valves (Johnson et al., 2006). BMP2 and BMP4 activates its downstream targets via the phosphorylation of smad1/5/8 proteins (Derwall et al., 2011; Miller et al., 2010) and upregulate osteogenic transcription factors, Msx2, Runx2, and osterix (Franceschi & Xiao, 2003; Tyson et al., 2003).

Aforementioned, diseases such as CAVD may also be influenced by increased ROS production and decreased of NO bioavailability with aging (Miller et al., 2010). **The major gap in the research is our understanding of the**

mechanisms behind ROS production that contribute to endothelial dysfunction and valve disease with aging. Donato et al (2007) demonstrated that healthy individuals' endothelial function is impaired with age and associated with endothelial oxidative stress. However, this study did not provide a mechanism for increased ROS and endothelial dysfunction.

Research surrounding age-related genes, called Sirtuins, have been given massive amount of attention in the last five years, as a potential key to pathophysiological changes associated with age. Thus far there have been seven sirtuin isoforms identified. Down regulation of these genes has been shown to increase oxidative stress, reduce NO bioavailability, and shorten lifespan (Mostoslavsky et al., 2006). For instance, SIRT1, SIRT6, and SIRT7 knockout mice survive for a very short time and develop a progeroid phenotype at a very young age (Mostoslavsky et al., 2006). Largely, the role of sirtuins (SIRT) is to promote genomic stability via the deacetylation of histones. It has been investigated that some SIRTs, specifically SIRT3 (which is localized to the mitochondria), may protect against oxidative stress via upregulating MnSOD activity (Qiu, Brown, Hirschey, Verdin, & Chen, 2010). Donato and Seals et al have also provided evidence that 1) SIRT1 may protect endothelium against oxidative stress and 2) SIRT1 decreases with age, which may explain this observed endothelial dysfunction in healthy individuals (Donato et al., 2011). Currently, we are not aware of research examining SIRT expression in cardiac valves.

In this study, the examination of SIRT levels may provide a novel link between histone acetylation, RI-RR phenomenon, and endothelia function with increasing age.

So we hypothesize, that impaired endothelial and valve function with increasing age is the result of reductions of sirtuins and subsequent induction of ROS-induced ROS-release (See Figure 2).

METHODS

Controlled Experiment

A controlled experimental design was the most effective way to investigate the effects of MnSOD-deficiency and increasing age on endothelial function in mice (Leedy & Ormrod, 2009). First, mice were divided into young and aged cohorts that allowed examination of changes in vascular function with age. Second, these groups were divided into additional sub-groups based on genotype (MnSOD wild-type and MnSOD heterozygote). This study design was the most suitable to determine interactions between the four groups.

Based on previous findings by other investigators, in which mitochondrial oxidative stress induces NAD(P)H oxidase derived ROS, vice versa, mice deficient in MnSOD (A. E. Dikalova, Bikineyeva et al., 2010) were used. Mice deficient in MnSOD have been shown to increase oxidative stress and for the purpose of this study it would be the most suitable model (Y. Zhang et al., 2009). MnSOD-deficient cohorts were generated based on the breeding scheme of $MnSOD^{+/+}$ crossed with a $MnSOD^{+/-}$ to provide litters of 1:1 for each genotype. MnSOD null mice were not generated due to previous work has shown that complete knockdown of MnSOD is lethal (Y. Li et al., 1995). These mice were crossed onto a C57/Bl6J background, which this background has been found to be the most suitable for studies of vasomotor function with aging (Andresen, Faraci, & Heistad, 2004; Brown et al., 2007). Mice were littermate matched for

experiments when possible and wild-type littermates were used as control when comparing across genotypes.

Mice were aged out to two different time points to measure an aging effect on vascular function and to investigate the effects of RI-RR on endothelial function with age. The first group, young mice, was aged out to 3 months of age. This is the age where young mice are classified as sexually matured, young adults (Brown et al., 2007). Old mice, the second group, were aged out to at least 18 months of age and no later than 22 months of age. Past investigators have used similar classification for aging (Brown et al., 2007). Mice were housed in climate controlled facilities under a 12 hour light/dark cycle with free access to water and normal chow (PicoLab Mouse Diet 5053).

Experiments

PCR Genotyping

At time of wean, mice were separated based on sex. A small piece of tail was collected from each mouse to perform PCR genotyping. DNA isolation followed based on the Invitrogen DNA isolation mini kit. Primers used are Primer 1 5'-TGTTCTCCTCTTCCTCATCTCC-3', Primer 2 5'- ACCCTTTCCAAATCCTCAGC-3', Primer 3 5'- TGAACCAGTTGTGTTGTCAGG-3', and Primer 4 5'-TCCATCACTGGTCACTAGCC -3' (Andresen et al., 2004) and were generated from the Advanced Technologies Genomics Center at Mayo Clinic, Rochester, MN. Thermal cycle conditions were optimized to determine satisfactory results. Thermal cycle conditions are as follows: hot start of 94° for 3 minutes, and the

PCR conditions for 40 cycles: 94° for 20 seconds, 63° for 30 seconds, 72° for 30 seconds. PCR genotyping of the mice provided an internal control that the correct genotype of each mouse is determined correctly and accurately.

Blood pressure

Blood pressure was measured in both in young and old cohorts, approximately, one week prior to sacrifice. Blood pressure was measured using tail-cuff pneumatic system (Kent Scientific). For five consecutive days, blood pressure was measured to allow the mice to acclimate to the process. The first three days were used as the acclimation period where the mice are to become more comfortable with the tail cuff procedure. The remaining two days were used for analysis. The purpose to measure blood pressure was to ensure no confounding variable was being observed (e.g. hypertension can induce endothelial dysfunction) (Rodriguez-Iturbe et al., 2007).

Sacrifice

Mice were sacrificed at the respective aged timepoints for instance, 3 months of age for the young cohort and approximately 18 months of age for the old group. Prior to sacrifice, mice were fasted overnight as we have found in pilot studies that not fasting prior to sacrifice causes high variability in glucose readings and vascular function. At time of sacrifice mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (75-100mg/kg) (Miller, Peotta et al., 2008). The aorta was carefully dissected out to ensure the endothelial and smooth muscle is not damaged. The aorta was then placed in cold Krebs

solution to have any adventitia tissue and perivascular fat removed. The descending thoracic aorta was sectioned in four 2-3 mm lengths for vasomotor function study, whereas the arch was used for quantitative-RT PCR for the measurement of gene expression. A small segment of aorta (1-2mm in length) was used for immunohistochemistry.

Vasomotor Function Study

Miller et al along with other investigators have used the following protocol as a way to measure aortic isometric tension and relaxation (Andresen et al., 2004; Brown et al., 2007; Miller, Peotta et al., 2008). The four small segments of aorta were placed onto two stainless steel triangular hooks. Organ chamber baths were set at physiological ambient temperature at 37°C and oxygenated with 95% O_2 and 5% CO_2 mixture. Organ chambers were filled with Krebs solution to provide vessels with nutrients to be viable throughout the study. Computer software from ADI Instruments recorded changes in force. Vessels suspended in the baths, and were periodically tightened to 0.6 grams of tension for approximately 1.5 hours until all are stabilized. At this time, 0.6 grams became the baseline for the future experimental curves. Vessels were given in log doses of serotonin (5-HT 10^{-8}M—10^{-5}M; Sigma-Aldrich, St. Louis, MO) to measure contraction forces. After the maximum contraction, vessels were rinsed with fresh Krebs solution. Approximately, after 30 minute incubation, vessels were preconstricted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) to approximately 60-70% of maximum force. After the vessels plateaued, log doses of acetylcholine (Ach, 10^{-}

^{9}M—10^{-4}M, Sigma-Aldrich; St. Louis, MO), endothelium-dependent relaxation, were given to each bath at 60 second intervals. Relaxation curve to sodium nitroprusside (SNP, 10^{-9}M—10^{-4}M, Sigma-Aldrich; St. Louis, MO), endothelium-independent relaxation, was also measured. Maximum constriction force of the vessels was measured based on doses of $PGF_{2\alpha}$ (10^{-7}M—10^{-4}M), before the proceeding dose was administered plateau of the vessels was achieved to gain a true representation of maximum force.

To measure functional consequences of various inhibitory drugs, vessels were pre-treated with drugs prior to each acetylcholine curve. Two sections of the four total sections were used as a control and the other two for drug incubation for each mouse. To measure effects of inhibition of NAD(P)H oxidase, treated baths were given 100μM (Hamilton et al., 2002) of apocynin and incubated for a total of 30 minutes. For specific Nox2 inhibition, 500nM (Rey et al., 2001)of gp91dstat unscrambled peptide 2 was administered in the treated baths, and since gp91dstat peptide 2 comes in two different isoforms (scrambled/unscrambled) the control vessels were given the scrambled peptide of gp91dstat 2. Both were incubated for equal time of 30 minutes. There are two reasons for incubating the vessels with gp91dstat 1) to determine if ROS production is the result of Nox2 in the cell and 2) apocynin has been recently controversial that it could not be a NAD(P)H oxidase inhibitor but rather have antioxidant properties (Heumuller et al., 2008).

To analyze the results from the vasomotor function study, Lab Chart software by ADI Instruments will be used. The results were tabulated in Microsoft Excel and displayed on charts that will have the independent variable (x-axis) dose of Ach, SNP, or PGF2α, and % relaxation as the dependent variable (y-axis).

Gene Expression

Gene expression was measured from the aortic arch in both young and old animals. Aortic arch was placed in 300μL of RNA lysis buffer, 1% β-mercapaethanol mixture. Extraction of RNA was obtained using Invitrogen RNA isolation mini kit. After RNA isolation, reverse transcriptase was performed on the RNA to produce cDNA (VILO synthesis, Invitrogen). Equal amounts of RNA loaded were loaded into each reverse transcriptase reaction, to control for variability across isolations. RNA loaded into RT reactions were based on RNA concentrations that was isolated (Nanodrop 2000, FisherScientific). Quantitiative Real Time RT-PCR was performed on predetermined genes using a StepOne Plus instrument (Applied Biosystems) and changes in ΔΔCT were measured. As an internal control, an appropriate housekeeper was chosen to normalize the gene of interest to the housekeeper, again, to control for variability.

The genes of interest can be divided into four main groups: SOD's, NAD(P)H oxidases, NOS isoforms, and age-related genes. MnSOD, CuZnSOD, and ecSOD were measured 1) to ensure our intervention by reducing MnSOD was reduced ~50% in heterozygote mice and 2) there is not a compensatory mechanism occurring due to reductions of MnSOD. Second group of gene

expression data investigated genes that regulate antioxidant genes (Nrf2, FOXO1, FOXO3, FOXO4, DJ-1, and Nqo1). Third group of gene expression measured are pro-oxidants: Nox1, Nox2, Nox4, and $p47^{phox}$ (necessary subunit of Nox2). We did not measure Nox5 and Nox3 as that is not found in the species of mice and specifically only expressed in the brain, respectively (Drummond et al., 2011). Third, NOS isoforms, endothelial NOS (eNOS), inducible NOS (iNOS), and neuronal NOS (nNOS) were measured. Increases of iNOS is often associated with inflammatory increases and oxidative stress (Csiszar et al., 2002; Csiszar et al., 2003). Fourth, pro-osteogenesis genes were measured, Runx2, Msx2, and SP7. Finally, age-related genes, the sirtuin isoforms (SIRT1-7) and Werner protein, were measured to determine if there was a reduction of any longevity gene expression levels with age or with MnSOD deficiency.

Immunohistochemistry

At time of sacrifice small segment of aorta was taken and embedded in OCT. After the tissue was embedded in OCT, 10μm sections were taking using a cryostat machine. Sections were adhered to glass slides, consequently, were stained for global acetylation lysines and Histone 3 Lysine 9 (H3K9). After staining, sections were imaged using confocal microscopy (Zeiss 780). Images were analyzed by ImageJ software.

Cardiac and aortic valve function

Cardiac and aortic valve function were assessed using echocardiography (GE Vivid 7 ultrasound system, 13 MHz linear probe) under light isoflurane anesthesia

to avoid anesthesia-induced cardiodepression (heart rates were maintained above >475 beats/minute). Cardiac function was evaluated from parasternal short-axis images at the level of the papillary muscles. Aortic valve cusp separation distance was measured from M-mode images taken from the parasternal long-axis view, and peak transvalvular velocities were measured with Doppler velocimetry from the suprasternal notch view.

Ethical Considerations

All animal experiments and protocols were approved by the Institutional Animal Care and Use (IACUC) at Mayo Clinic, Rochester, Minnesota.

Validity

This study was closely controlled by the use of controls, normalization, and validation. Necessary controls were used in each experiment. A way to control for variability across the mice, is that we littermate matched the mice if at all possible to eliminate possible variation between litters and breeders. During experiments, wild-type was used as the control when comparing across genotypes and young mice, matched with their respective genotype, was used as a control when observing an age effect. Calibration of the vasomotor function equipment was performed prior to each experiment. The treatment of vessels during vasomotor function studies, organ chambers were divided into two groups (treated or non-treated) to observe a change and to have a time control bath. For quantitative real-time RT PCR, housekeeping genes were used to control for variation in the quality and quantity of RNA samples.

Statistical Analysis

All data are expressed in SE±. Main effects of age, genotype, and age-by-genotype interactions were detected using two-way ANOVA testing. Subsequent comparisons across groups were made using Bonferroni-corrected t-tests. Based on previous preliminary data, we anticipated that 15 animals per group would be sufficient to detect change with pre-determined power >0.80 when $\alpha=0.05$.

RESULTS

Antioxidant gene expression in aorta. As expected, expression of MnSOD was reduced by ~50% in $MnSOD^{+/-}$ mice compared to $MnSOD^{+/+}$ mice in both young and old groups. MnSOD-deficiency did not affect expression of CuZnSOD and ecSOD in young mice, nor did it affect age-related reductions in these genes (Figure 3A-C).

Antioxidant gene expression in aortic valve. As expected, expression of MnSOD was reduced by ~50% in aortic valves from $MnSOD^{+/-}$ mice compared to age-matched $MnSOD^{+/+}$ mice. Expression of CuZnSOD and ecSOD was markedly reduced by aging, and these reductions were not affected by MnSOD deficiency (Figure 3D-F).

Genes regulating antioxidant responses in aorta. Expression of FOXO1, FOXO3, and FOXO4 were significantly reduced with aging, but these changes were not altered by MnSOD-deficiency. Nrf2 was slightly decreased with age but not affected by MnSOD-deficiency. Nqo1 and DJ-1 were relatively unchanged across both age and genotype groups (Figure 4A-B and Appendix 1A-D).

Genes regulating antioxidant responses in aortic valve. Expression of FOXO1, FOXO3, and FOXO4 was reduced in aortic valve with aging and, similar to aorta, these changes were not altered by MnSOD-deficiency. Reduction of MnSOD also did not affect age-related declines in Nrf2 or DJ-1 (Figure 4C-D and Appendix 1E-H).

Pro-oxidant and pro-inflammatory gene expression in aorta*.* Similar to $MnSOD^{+/+}$ mice, expression of Nox1 was not detectable in aorta from $MnSOD^{+/-}$ mice, and expression of Nox4 was not altered with aging in either group (Figure 5B). Expression of Nox2 in aorta was elevated in both young and old $MnSOD^{+/-}$ mice compared to age-matched $MnSOD^{+/+}$ mice (Figure 5A), and was paralleled by changes in $p47^{phox}$ expression (Appendix 2A). Changes in expression of TNF-α were similar between $MnSOD^{+/+}$ and $MnSOD^{+/-}$ mice within and across age groups (Appendix 3C).

Pro-oxidant and pro-inflammatory gene expression in aortic valve. In aortic valves from $MnSOD^{+/-}$ mice, Nox4 was decreased by aging to a similar extent to that observed in $MnSOD^{+/+}$ mice (Figure 5D). Unlike aorta, however, expression of Nox2 was not increased in $MnSOD^{+/-}$ mice compared to age-matched $MnSOD^{+/+}$ mice (Figure 5C). Expression of ICAM, VCAM, and TNFα in young and aged $MnSOD^{+/-}$ mice were similar to levels observed in their age-matched $MnSOD^{+/+}$ littermates (Appendix 3D-F).

Changes in NOS isoform expression in aorta**.** In aorta, eNOS expression was not affected by aging in $MnSOD^{+/+}$ or $MnSOD^{+/-}$ mice. Similarly, expression of iNOS was not significantly affected by aging in $MnSOD^{+/+}$ or $MnSOD^{+/-}$ mice (Table 1).

Changes in NOS isoform expression in aortic valve. MnSOD-deficiency did not alter eNOS expression in aortic valves from young mice, and $MnSOD^{+/-}$ mice had similar age-related reductions in eNOS compared to $MnSOD^{+/+}$ littermates.

Age-related increases in iNOS expression in aortic valve were similar between $MnSOD^{+/+}$ and $MnSOD^{+/-}$ mice. Expression of nNOS was not detectable in young or aged $MnSOD^{+/-}$ mice (Table 1).

Aging- and senescence-related gene expression in aorta. There were significant age-related decreases of SIRT1, SIRT2, and WRN expression in aorta (Figure 6A-B and Appendix 4A). SIRT5, SIRT6, and SIRT7 were significantly increased with age, but were not affected by reduction of MnSOD (Figure 6E-G). Expression of cyclin-dependent kinase inhibitor 2a (CDKN2A) was significantly increased in aged mice, but was not affected by reduction of MnSOD (Figure 6H).

Aging- and senescence-related gene expression levels in aortic valve. Sirtuin expression was not affected by MnSOD deficiency in young mice, and age-related reductions in SIRT1, SIRT2, SIRT3, SIRT4, SIRT6, and Wrn (Figure 6I-L, N and Appendix 4B) were similar in aortic valves from $MnSOD^{+/-}$ and $MnSOD^{+/+}$ mice. Expression of SIRT5, however, was significantly increased in aged $MnSOD^{+/-}$ mice compared to young $MnSOD^{+/-}$ mice (Figure 6M). Expression of CDKN2A was profoundly increased in aged mice and was not affected by MnSOD deficiency (Figure 6P).

Vasomotor Function Results

Vascular relaxation to acetylcholine was significantly reduced in aged $MnSOD^{+/+}$ mice compared to young $MnSOD^{+/+}$ mice. Inhibition of NAD(P)H oxidase with apocynin did not affect responses to acetylcholine in young

$MnSOD^{+/+}$ mice, and significantly improved responses to acetylcholine in aged $MnSOD^{+/+}$ mice (Figure 7A-B). Responses to sodium nitroprusside were unaffected by aging (Table 2). Maximum tension in response to $PGF_{2\alpha}$ was augmented in aged $MnSOD^{+/+}$ mice compared to young $MnSOD^{+/+}$ mice (Table 2).

Relaxation to acetylcholine was not impaired in aorta from young $MnSOD^{+/-}$ mice compared to $MnSOD^{+/+}$ littermates, and age-related impairments in endothelial function were similar in aorta from the two genotypes of mice. In contrast, NAD(P)H oxidase inhibition significantly impaired responses to acetylcholine in both young and old $MnSOD^{+/-}$ mice (Figure 7A-B). Similar responses were observed following incubation with gp91ds-TAT, an inhibitor of the Nox2 catalytic subunit (Appendix 5). Responses to sodium nitroprusside were similar between $MnSOD^{+/-}$ and $MnSOD^{+/+}$ mice in both age groups (Table 2). Responses to $PGF_{2\alpha}$ in $MnSOD^{+/-}$ mice were similar to those observed in $MnSOD^{+/+}$ mice in both age groups (Table 2).

Aortic Valve Function

Aortic valve cusp separation was not significantly different between young and old $MnSOD^{+/+}$ mice, and were not altered by MnSOD haploinsufficiency (Figure 7C). Similarly, peak transvalvular velocity was largely unchanged between groups (Figure 7D). We also did not detect differences in the prevalence of aortic valve regurgitation between the two groups.

Cardiac Function

Heart rates under anesthesia were maintained >500 bpm and there were no differences between groups (Appendix 6A). Ejection fraction was significantly reduced with aging in both genotypes (Appendix 6B). End-diastolic dimensions and end-systolic dimensions were significantly increased in aged mice MnSOD$^{+/+}$ mice, and these changes were not affected by MnSOD haploinsufficiency (Appendix 6C-D).

DISCUSSION

The main findings of this study are: 1) there are dramatically different transcriptional responses to aging in aorta and aortic valve, 2) the functional consequences of NAD(P)H oxidase-derived radicals are critically dependent on mitochondrial antioxidant levels in aorta in both young and aged mice, 3) despite apparent deleterious molecular changes, aortic valve function is remarkably well preserved with increasing age in mice, and 4) reductions in mitochondrial antioxidant capacity amplify and modulate, but do not independently initiate, osteogenic signaling in vascular smooth muscle cells.

Transcriptional responses to aging in aorta

In aorta from wild-type mice, we found that aging was associated with changes in transcriptional patterns that favored a pro-oxidative environment. Specifically, levels of ecSOD and CuZnSOD were significantly reduced and expression of Nox2 was significantly increased in aorta from aged wild-type mice. This reduced antioxidant capacity is consistent with data from previous reports in aging mice, and may be in part due to age-associated reductions the transcription factors DJ-1 and members of the FOXO family, ultimately resulting in reductions in Nrf2 (Collins et al., 2009; Ungvari et al., 2011). NAD(P)H oxidase-derived reactive oxygen species have also been shown to be increased with aging, and have also been shown to play a key role in limiting nitric oxide bioavailability (Hamilton et al., 2002; Podlutsky et al., 2010) and promoting fibrosis in aged conduit vessels (Lakatta et al., 2009). When combined with age-

related reductions in endothelial nitric oxide synthase and SIRT1 expression, these molecular changes would be expected to strongly favor perivascular fibrosis and vascular stiffening with aging (Donato et al., 2011).

In the present study we found that Nox2 expression was increased in young and old MnSOD-deficient mice compared to their wild-type littermates. This finding is consistent with work from previous work in cultured endothelial cells demonstrating a positive feedback loop between mitochondria and NAD(P)H oxidase-derived ROS (A. E. Dikalova, Bikineyeva et al., 2010)—a phenomenon more commonly referred to as ROS-induced ROS release. This pro-oxidative environment did not, however, augment age-related reductions in expression of endothelial nitric oxide synthase or sirtuin enzymes, nor did these changes augment age-associated increases in pro-fibrotic or pro-calcific gene expression (see online supplement). Thus, these data provide compelling evidence against the “oxidative stress hypothesis of cardiovascular aging” (Pedruzzi et al., 2004), and instead support a working model in which ROS-induced ROS-release initiated by losses in mitochondrial antioxidant capacity is not a primary driver of molecular changes promoting age-related vascular disease, and are instead likely to alter vasomotor function in a highly context-dependent manner in aorta.

Transcriptional responses to aging in aortic valve

Similar to our findings in aorta, our data from aortic valves suggest that age-related reductions in antioxidant expression in wild-type mice also favor a pro-oxidative environment. Unlike aorta, however, expression of NAD(P)H oxidase

subunits was not increased with aging, and expression of Nox4 was significantly decreased. To our knowledge, these are the first data characterizing changes in pro- and anti-oxidant enzymes in aged (but functionally normal) aortic valves, and the first to identify a fundamental difference in expression of redox-related genes in aorta and aortic valve. While these age-related reductions in antioxidant gene expression are remarkably similar to those observed in stenotic aortic valves (Miller, Chu et al., 2008), we did not observe increases in pro-fibrotic or pro-calcific gene expression in aortic valves from aged MnSOD$^{+/+}$ mice. Taken together with our data from aorta, it is possible that activation of pro-fibrotic and pro-calcific signaling is linked to NAD(P)H oxidase activation in wild-type mice. This postulate is consistent from findings in myocardium, where upregulation of Nox4 appears to be a critical regulatory event in the induction of pro-fibrotic signaling cascades (Rey et al., 2001), and from data in aorta, where administration of exogenous antioxidants reduces NAD(P)H oxidase-derived ROS and attenuates pro-fibrotic signaling (Cai & Harrison, 2000; Fleenor, Seals, Zigler, & Sindler, 2011).

The lack of an increase in pro-fibrotic and pro-calcific gene expression in aortic valves from MnSOD$^{+/+}$ mice was particularly surprising given the observation that expression of multiple sirtuin isoforms was markedly reduced in aortic valves with aging. Sirtuin deacetylases are emerging as important regulators of gene expression and function in the cardiovascular system. In particular, SIRT1 has been shown to inhibit TGFβ-induced gene expression and fibrosis in aorta and

myocardium with aging (Roger et al., 2011), and has been shown to be critical for maintenance of nitric oxide synthase function (A. Dikalova et al., 2005; Hamilton et al., 2002). Collectively, age-associated induction of valvular fibrosis and calcification may well involve a complex interplay and balance between activation of TGFβ superfamily signaling, increases in NAD(P)H oxidase-derived ROS, and reductions in sirtuin levels.

In aortic valves from $MnSOD^{+/-}$ mice, we did not observe significant changes in NAD(P)H oxidase expression. Importantly, these are the first data suggesting that mitochondria-initiated ROS-induced ROS release may occur in a highly tissue specific manner, does not appear to occur in the aortic valve, and represents a fundamental difference in the molecular responses to aging and increased mitochondrial oxidative stress in aorta and aortic valve. Similar to our findings in aorta from $MnSOD^{+/-}$ mice, however, MnSOD deficiency did not elicit further reductions in sirtuin expression or increases in pro-fibrotic signaling in aortic valves from young or aged mice. Collectively, these data suggest that reductions in MnSOD are not likely to be a primary driver of pro-fibrotic or pro-calcific gene expression in aortic valves from young or aged mice, and instead may play a modulatory or permissive role when pro-fibrotic or pro-calcific stimuli are present.

Functional consequences of aging and MnSOD deficiency in aorta

We observed significant reductions in endothelial function with aging in wild-type mice that were largely reversible by inhibition of NAD(P)H oxidase with apocynin

or the Nox2 inhibitor gp91ds-tat. This is consistent with previous work demonstrating that NAD(P)H oxidase (and Nox2-derived radicals in particular) are important contributors to age-related declines in endothelial function (Csiszar et al., 2002; Hamilton et al., 2002; Podlutsky et al., 2010).

Consistent with previous reports, we also found that endothelial function was not impaired in young $MnSOD^{+/-}$ mice compared to their $MnSOD^{+/+}$ littermates (Andresen et al., 2004; Brown et al., 2007). We were, however, somewhat surprised to find that endothelial function in aged mice was not further impaired by MnSOD haploinsufficiency, as other groups have reported greater impairment of endothelial function in aged $MnSOD^{+/-}$ mice (Andresen et al., 2004; Brown et al., 2007). One potential explanation for this difference in findings is that our "aged" mice were significantly younger than those used in previous studies (~18 months old versus 24+ months old). Differences in animal husbandry, cumulative environmental stress, and diet may also significantly impact metabolic state and endothelial function with aging (S. I. Dikalov et al., 2008).

A major novel finding of this study, however, is that inhibition of NAD(P)H oxidase significantly impaired endothelial function in both young and aged $MnSOD^{+/-}$ mice. This finding was contrary to our hypothesis that ROS-induced ROS-release would impair endothelial function in aorta. Numerous studies have shown that, under a variety of stressed conditions, Nox2 or mitochondria-derived radicals impair endothelial function via reductions in NO bioavailability (S. Dikalov, 2011; Donato et al., 2007; Landmesser et al., 2003). Conversely,

several studies have reported that mitochondria-derived H_2O_2 elicits vasodilation in coronary arteries (Forstermann, 2010; Furchgott et al., 1987; Gongora et al., 2006; Y. Zhang et al., 2009), and that Nox4-derived ROS may play a central role in maintaining nitric oxide bioavailability (Drummond et al., 2011). Taken together, our data support a working model in which the vasomotor consequences of increased hydrogen peroxide levels are dependent not only on the level of physiological stress an organism is facing, but also on mitochondrial antioxidant status.

Functional consequences of aging and MnSOD deficiency in aortic valve

Aortic valve function in wild-type mice was unchanged with increasing age. Somewhat surprisingly, MnSOD deficiency did not impact aortic valve function compared to wild-type mice in both age groups. We anticipated that losses in mitochondrial antioxidant capacity would negatively impact aortic valve function with aging, due to previous studies suggesting that increases in oxidative stress are strongly associated with calcification in tissue from humans with calcific aortic valve disease (Miller, Chu et al., 2008), and experimental data suggesting that increases in oxidative stress and reductions in nitric oxide bioavailability accelerate cardiovascular cell calcification *in vitro* (Arnal, Dinh-Xuan, Pueyo, Darblade, & Rami, 1999; Touyz & Briones, 2011). A possible explanation for the contrast in findings is that the mice used in this study were not exposed to significant exogenous stressors (e.g., high fat diet, hypertensive stress, etc.), and the combination of hypercholesterolemia and oxidative stress are needed to

induce aortic valve disease in aging mice (Bengtsson et al., 2003; A. E. Dikalova, Gongora et al., 2010; Niu et al., 2010).

Conclusions

Collectively, our data demonstrate that the transcriptional responses and phenotypes elicited by aging and alterations in mitochondrial antioxidant capacity differ dramatically between aorta and aortic valve. Furthermore, the functional consequences of NAD(P)H-derived ROS and ROS-induced ROS-release appear to be critically dependent upon mitochondrial antioxidant capacity in aorta from "unstressed" mice. These data support a working model in which age-related reductions in mitochondrial antioxidant capacity are an important modulator, but not primary driver, of age-related cardiovascular disease.

TABLES

Table 1. Effects of aging and MnSOD-haploinsufficiency on expression of nitric oxide synthase isoforms. ** Denotes significant main effect of age with p-value < 0.05. Values are means ± SE; n = 4-14 mice/group.

	Aorta				Aortic Valve			
	Young		Old		Young		Old	
	WT	HET	WT	HET	WT	HET	WT	HET
Nitric Oxide Synthase Isoforms								
eNOS	1.0±0.1	1.1±0.1	0.9±0.1	0.9±0.1	1.0±0.1	1.0±0.1	0.7±0.2**	0.7±0.1**
iNOS	1.0±0.3	0.9±0.3	1.9±0.7	1.4±0.3	1.0±0.3	0.95±0.3	3.7±0.8**	5.0±1.0**
nNOS	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Table 2. Effects of aging and MnSOD-haploinsufficiency on vasomotor function in aorta. ** Denotes significant main effect of age with p-value < 0.05. Values are means ± SE; n = 8-23 mice/group.

	Aorta			
	Young		Old	
	WT	HET	WT	HET
Relaxation	62±3%	62±1%	49±3%**	43±3%**
MR_{ACH}				
$EC50_{ACH}$				
MR_{SNP}	90±2%	91±2%	94±2%	96±1%**
Constriction				
Peak Tension ($PGF_{2\alpha}$)	1.2±0.04	1.1±0.06	1.78±0.1**	1.74±0.1**
Peak Tension (5HT)	0.68±0.07	0.68±0.07	0.90±0.04**	0.86±0.04**

FIGURES

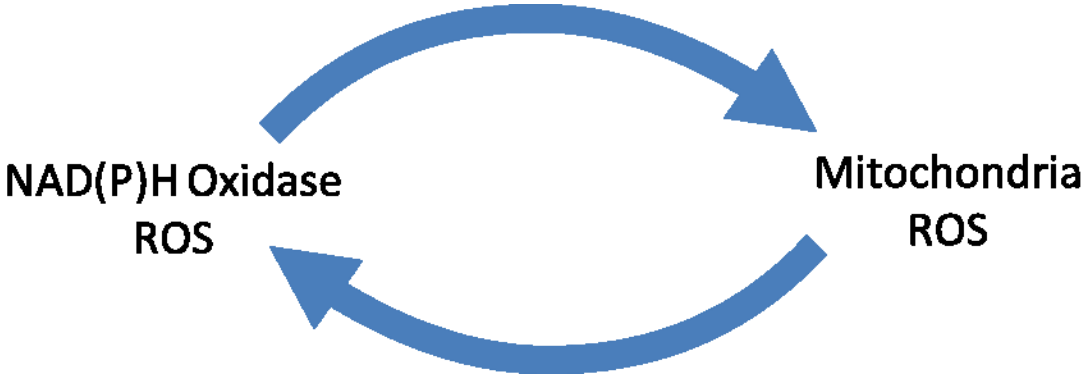

Figure 1 ***ROS-Induced ROS-Release Phenomenon***

In vitro studies have shown that production of ROS by NAD(P)H oxidase and mitochondria may self-perpetuate itself, creating a vicious cycle of ROS production.

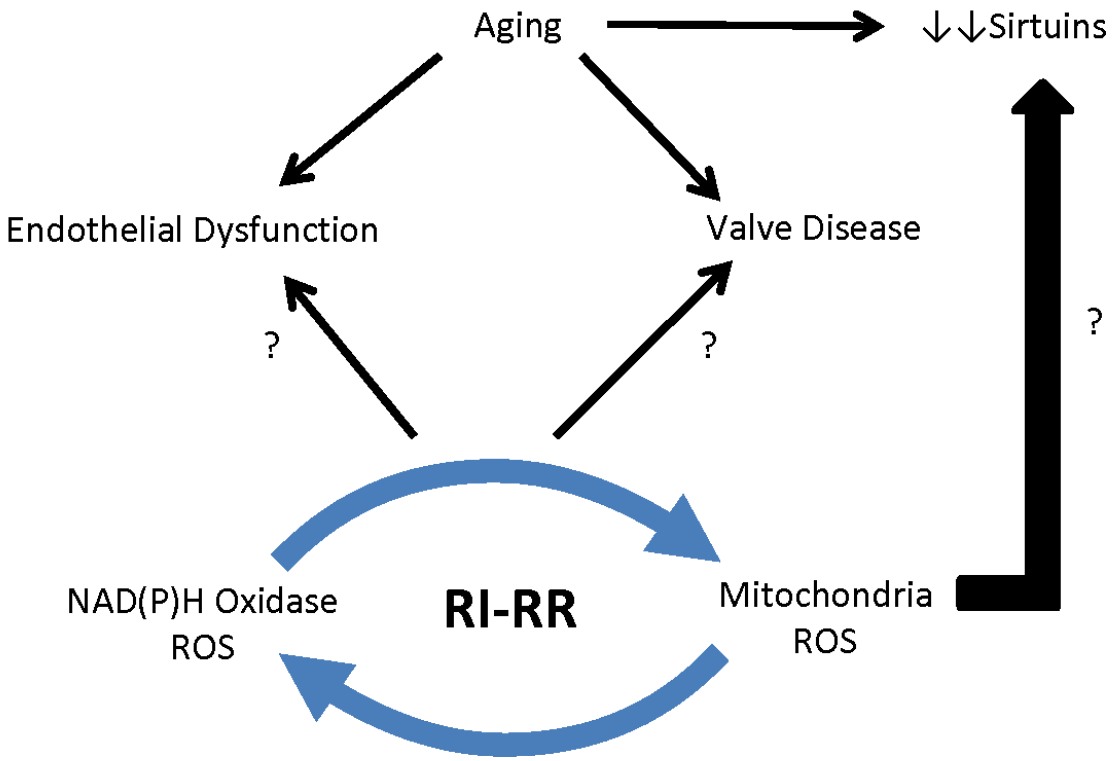

Figure 2: ***Working Model***

Aging is a major risk factor for various cardiovascular diseases, specifically, endothelial dysfunction and valve disease. Our hypothesis is that impaired endothelial and valve function with increasing age is the result of reductions of sirtuins and subsequent induction of ROS-induced ROS-release.

Figure 3: *Antioxidant gene expression levels legend*
(Fig 3A and 3D) MnSOD expression was reduced by ~50% in heterozygote mice ($p < 0.01$) in both aorta and aortic valve tissues. (Fig3B-C and Fig3E-F) There was no increase of other antioxidant mechanisms in both aorta and aortic valve due to reductions of MnSOD. However, CuZnSOD and ecSOD were significantly decreased with increasing age in both aorta and aortic valve. * Denotes significant main effect of genotype with p-value <0.05. ** Denotes significant main effect of age with p-value < 0.05. # Denotes significant differences between groups with p-value<0.05. Values are means ± SE; n = 4-14 mice/group.

Figure 3A: MnSOD expression in aorta.

Figure 3B: CuZnSOD expression in aorta.

Figure 3C: ecSOD expression in aorta.

Figure 3D: MnSOD expression in aortic valve.

Figure 3E: CuZnSOD expression in aortic valve.

Figure 3F: ecSOD expression aortic valve.

Composite of Figure 3.

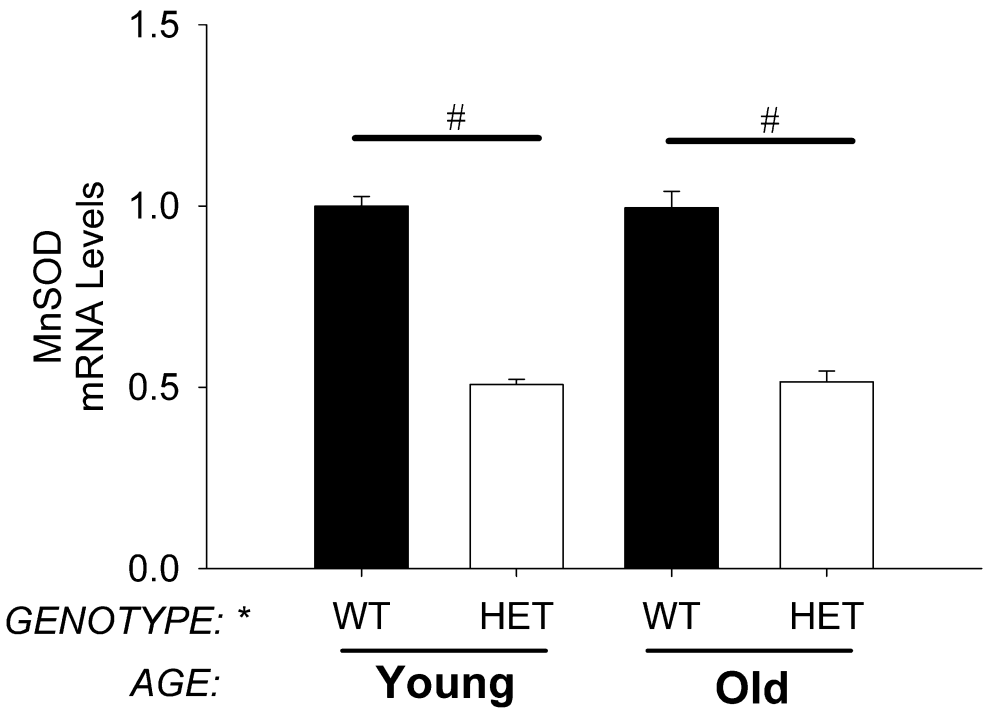

Figure 3A: ***MnSOD expression in aorta.***

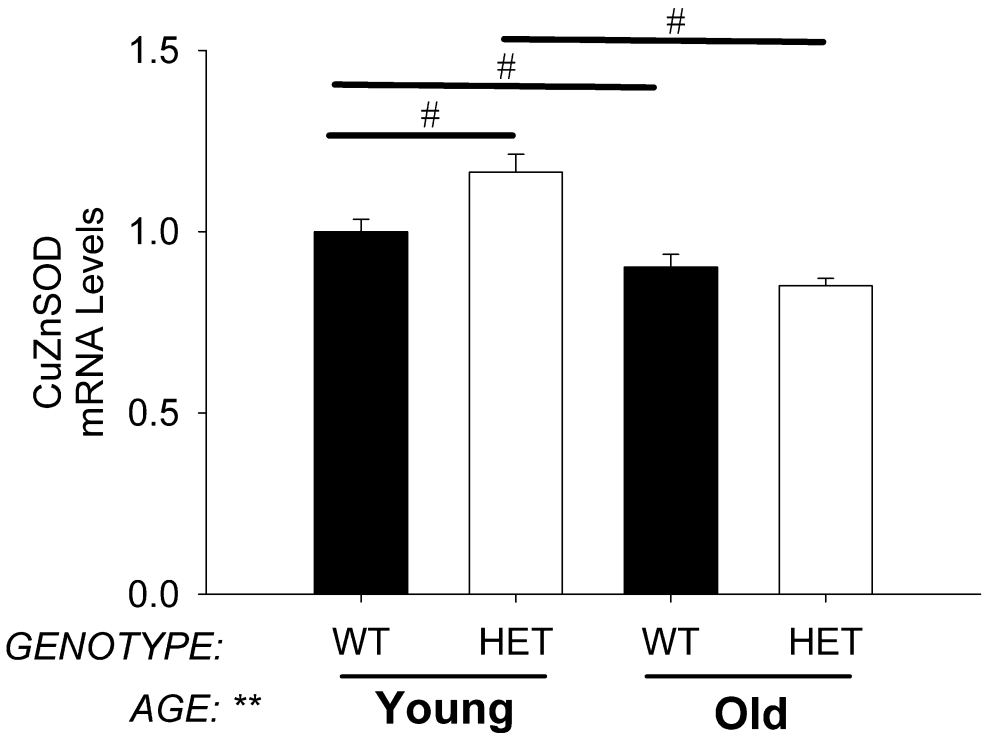

Figure 3B: ***CuZnSOD expression in aorta.***

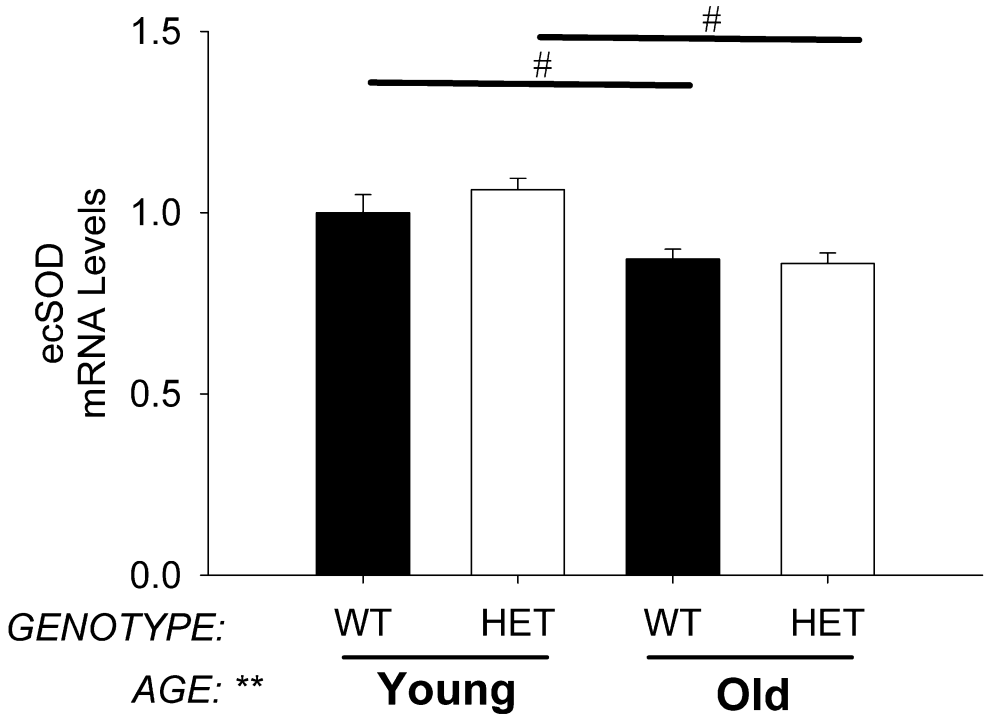

Figure 3C: ***ecSOD expression in aorta.***

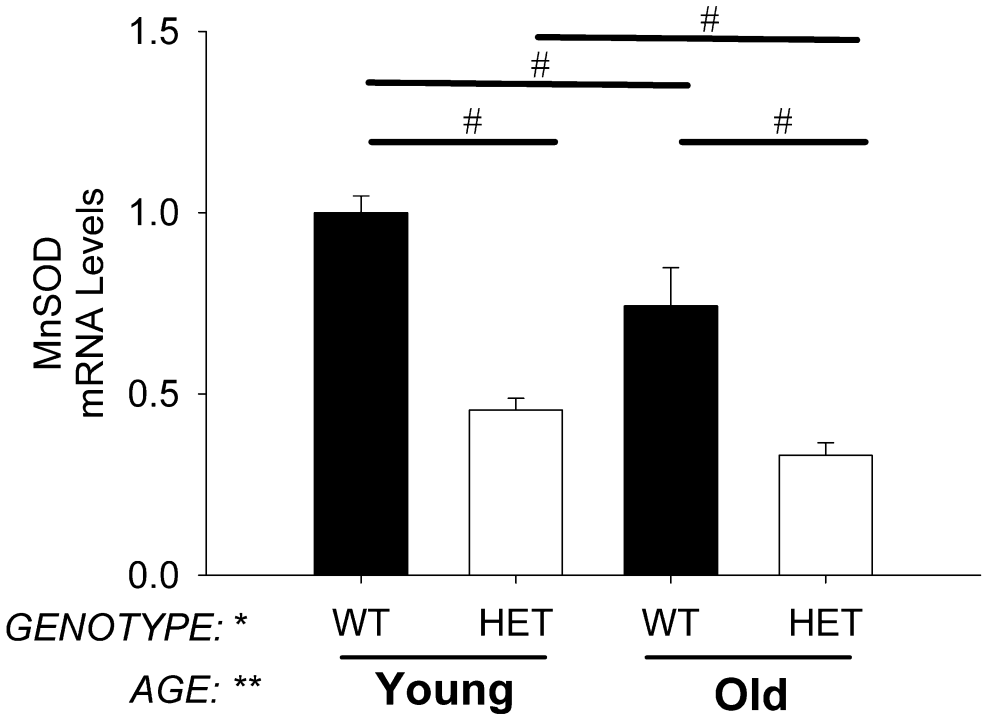

Figure 3D: ***MnSOD expression in aortic valve.***

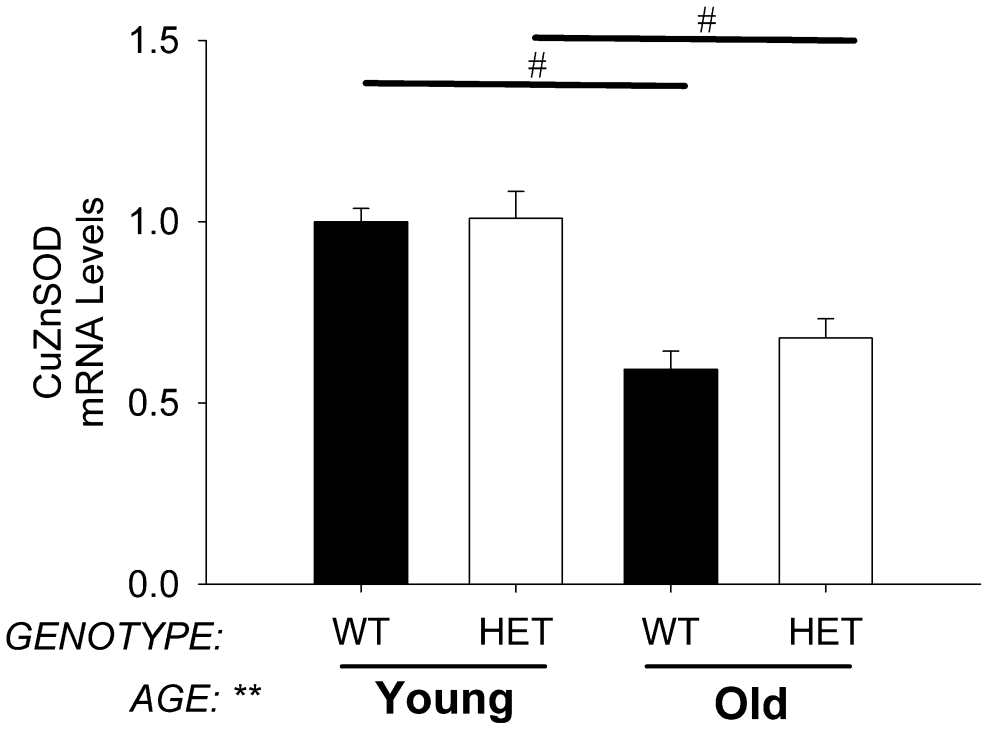

Figure 3E: ***CuZnSOD expression in aortic valve.***

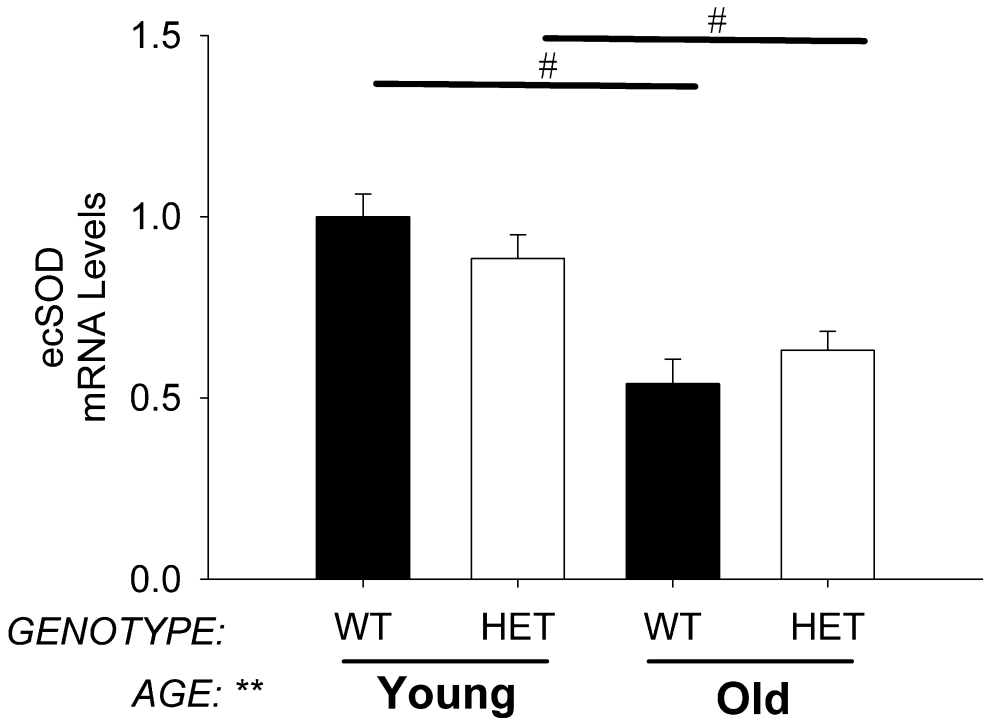

Figure 3F: ***ecSOD expression in aortic valve.***

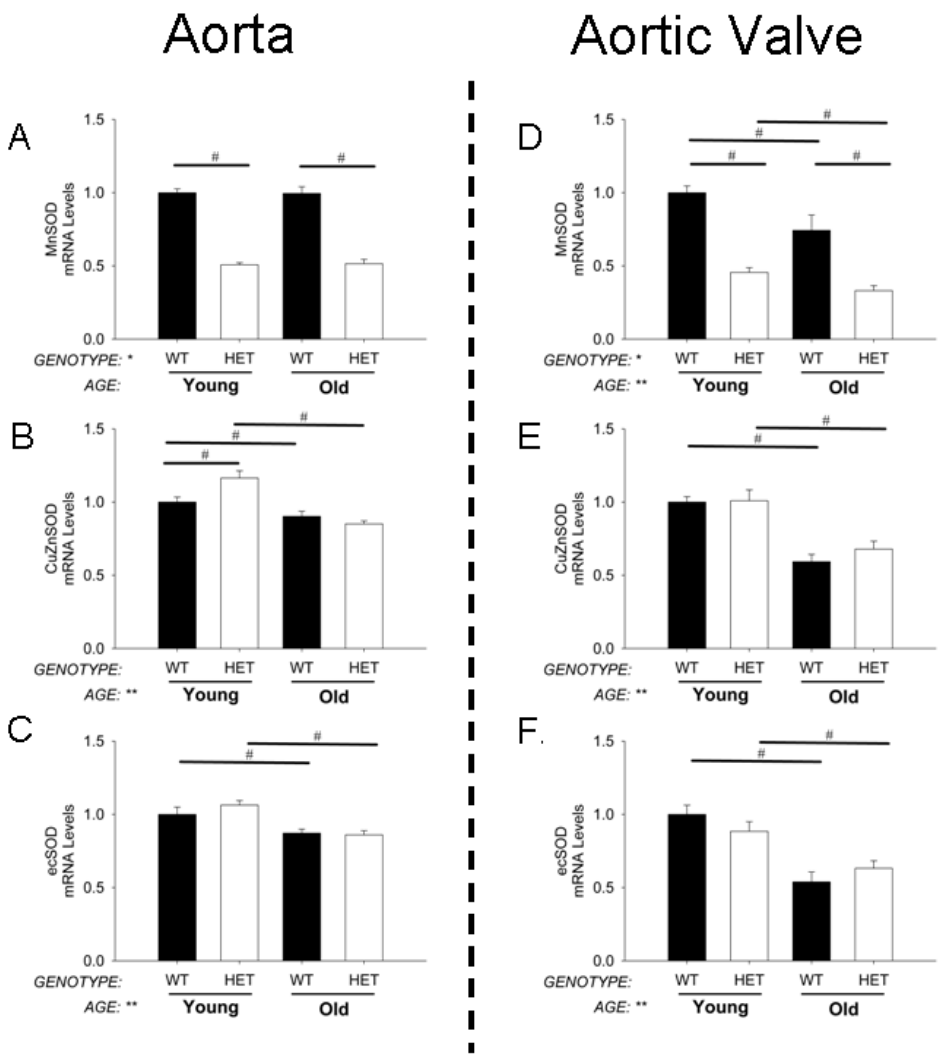

Figure 3: ***Composite***

.

Figure 4: ***Gene expression of genes regulating antioxidants legend*.**
Increasing age causes a significant decrease in FOXO-4 and Nrf2 gene expression, but MnSOD haploinsufficiency does not have an effect. . ** Denotes significant main effect of age with p-value < 0.05. # Denotes significant differences between groups with p-value<0.05. Values are means ± SE; n = 4-14 mice/group.

Figure 4A: FOXO-4 expression in aorta.

Figure 4B: Nrf2 expression in aorta.

Figure 4C: FOXO-4 expression in aortic valve.

Figure 4D: Nrf2 expression in aortic valve.

Composite of Figure 4.

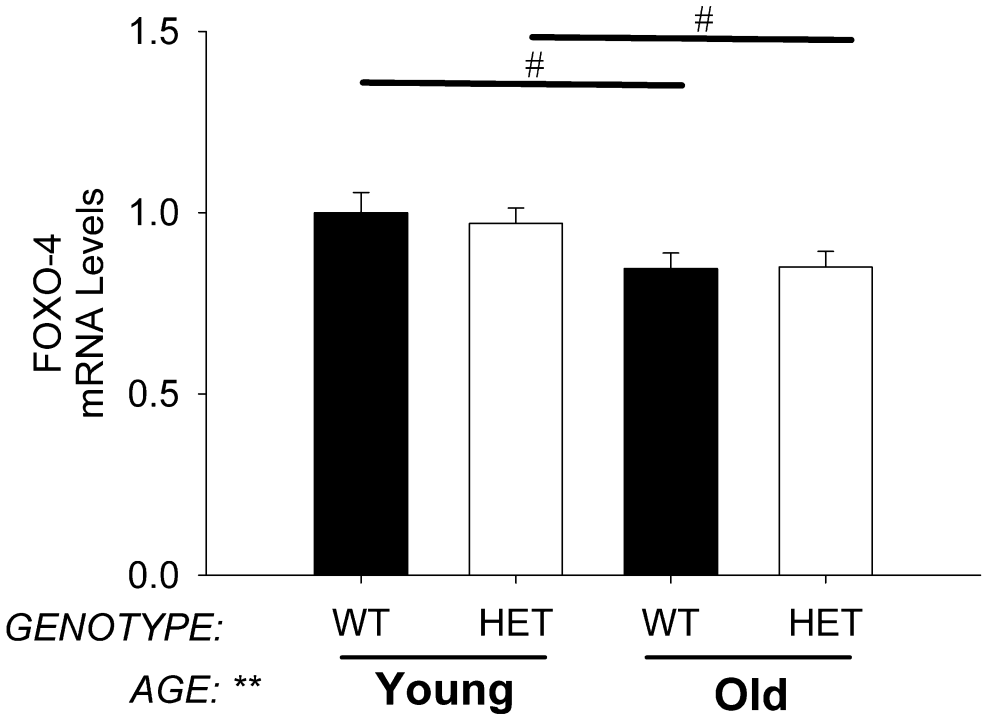

Figure 4A: ***FOXO-4 expression in aorta.***

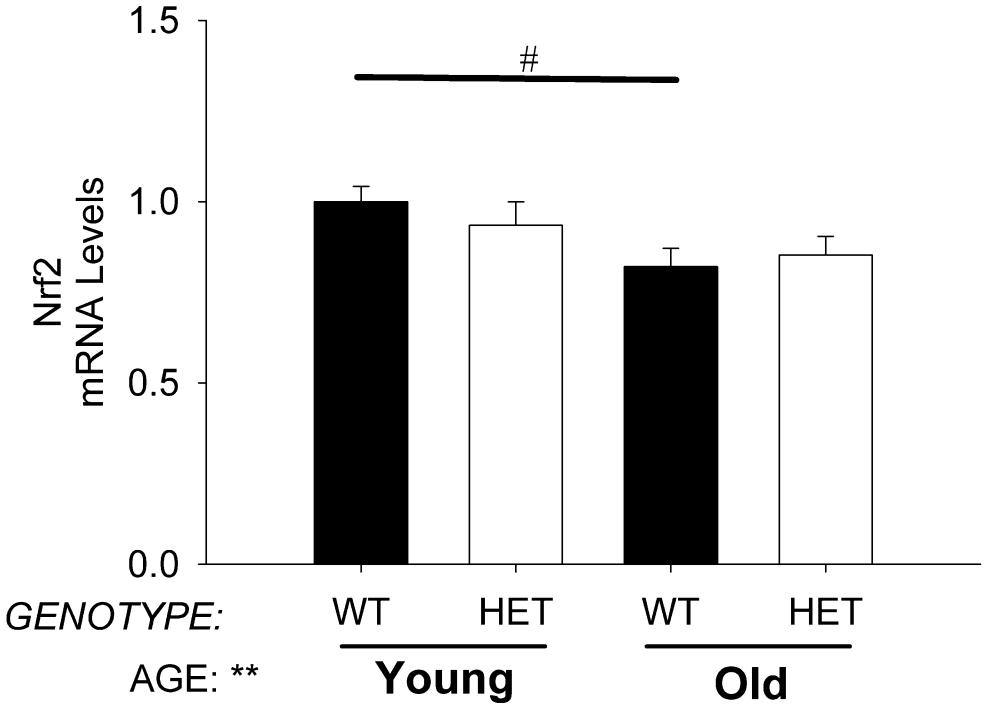

Figure 4B: ***Nrf2 expression in aorta.***

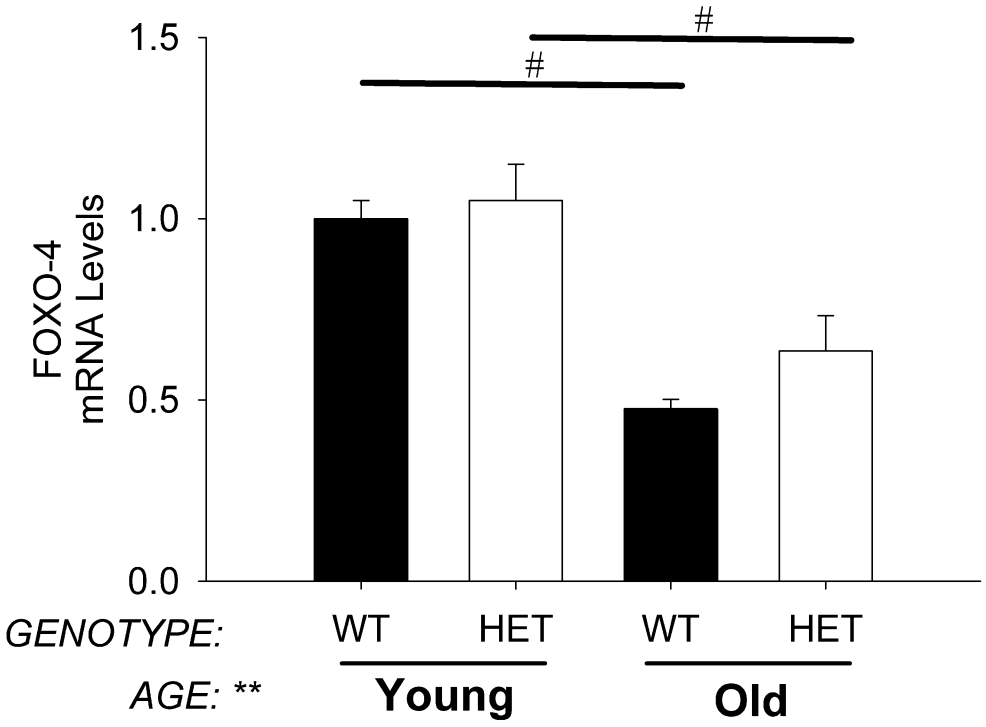

Figure 4C: ***FOXO-4 expression in aortic valve.***

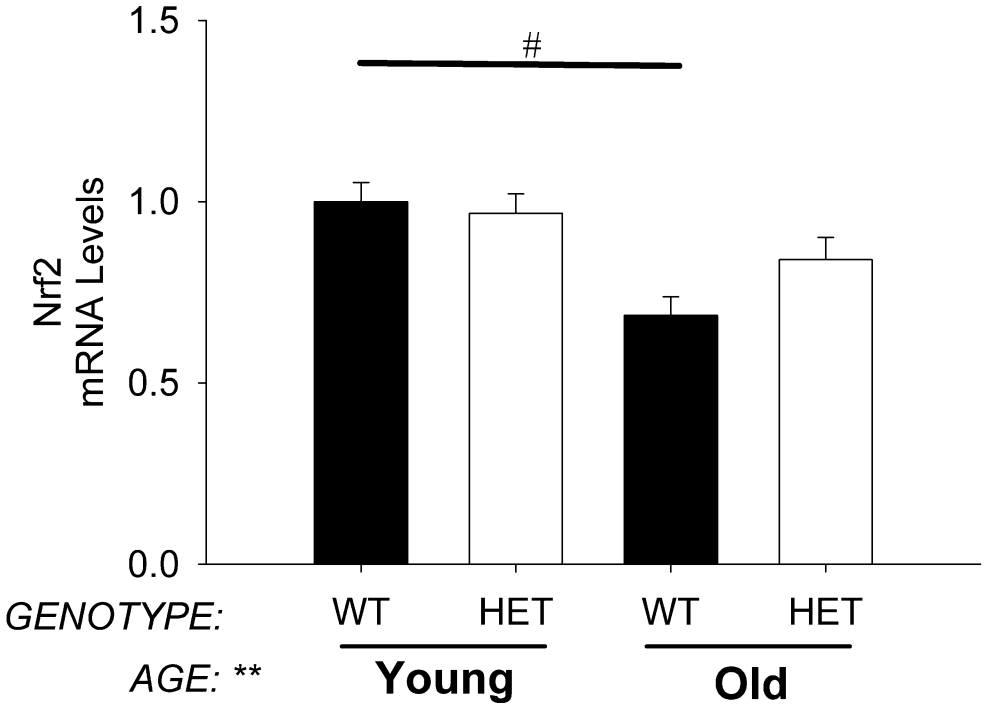

Figure 4D: ***Nrf2 expression in aortic valve.***

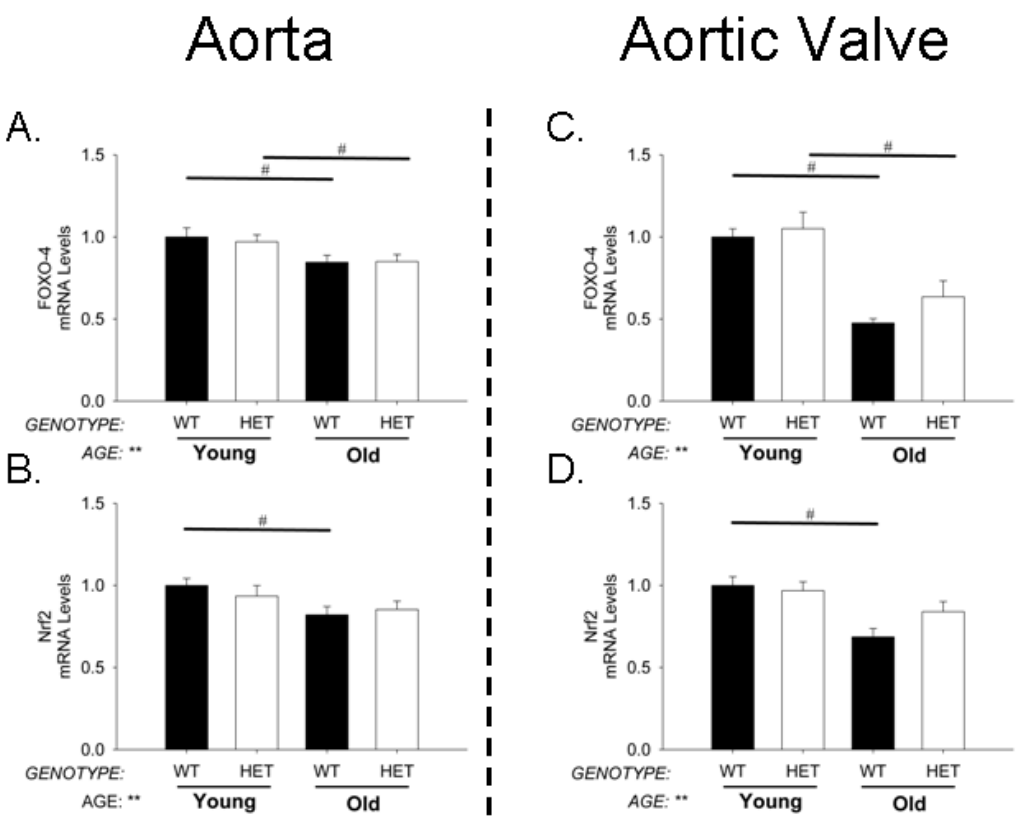

Figure 4: ***Composite***

Figure 5: ***Expression of NAD(P)H oxidase subunits legend.***
(A-D) Observe the large differences of Nox2 and Nox4 mRNA expression levels across tissues. *Denotes significant main effect of genotype with p-value <0.05. ** Denotes significant main effect of age with p-value < 0.05. # Denotes significant differences between groups with p-value<0.05. Values are means ± SE; n = 4-14 mice/group. Nox, NAD(P)H oxidase.

Figure 5A: Nox2 expression in aorta.

Figure 5B: Nox4 expression in aorta.

Figure 5C: Nox2 expression in aortic valve.

Figure 5D: Nox4 expression in aortic valve.

Composite of Figure 5.

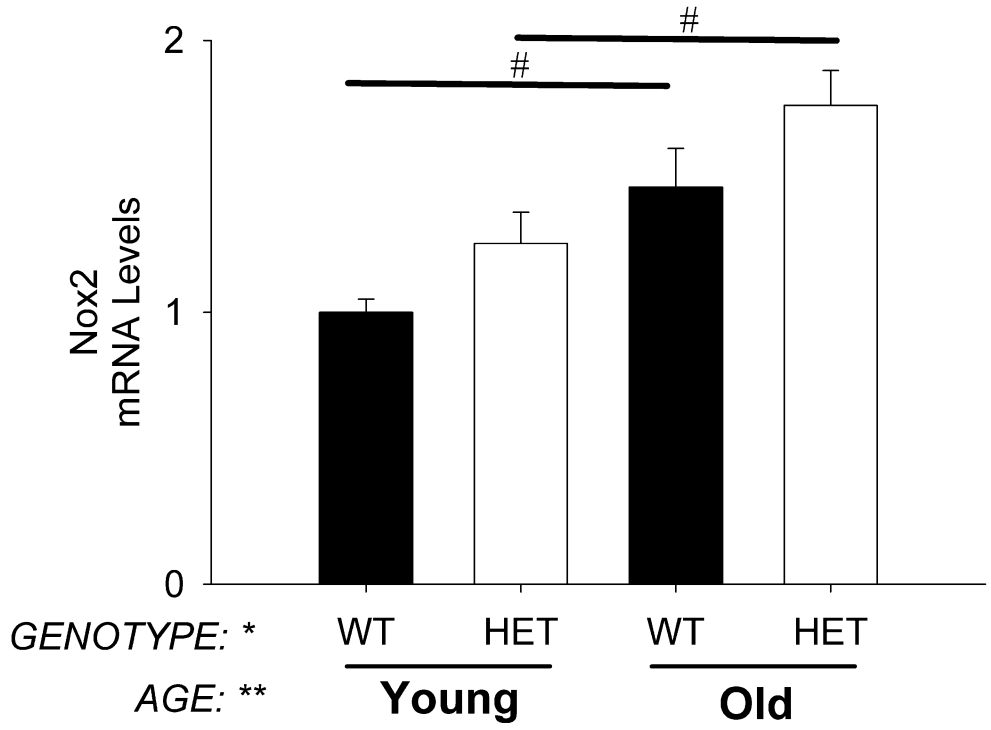

Figure 5A: ***Nox2 expression in aorta.***

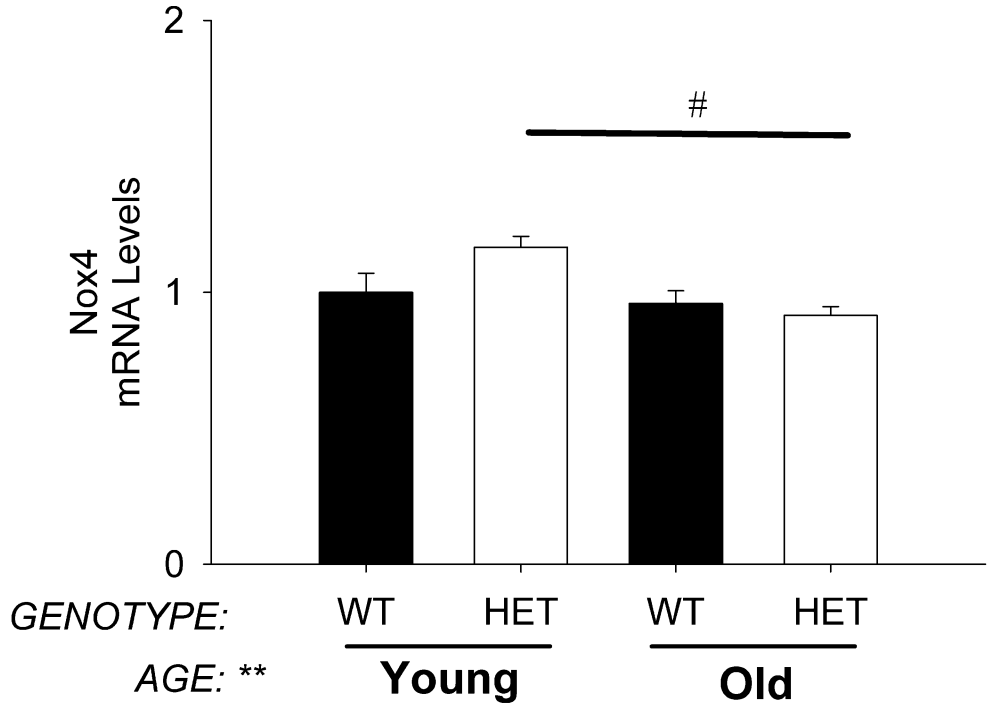

Figure 5B: ***Nox4 expression in aorta.***

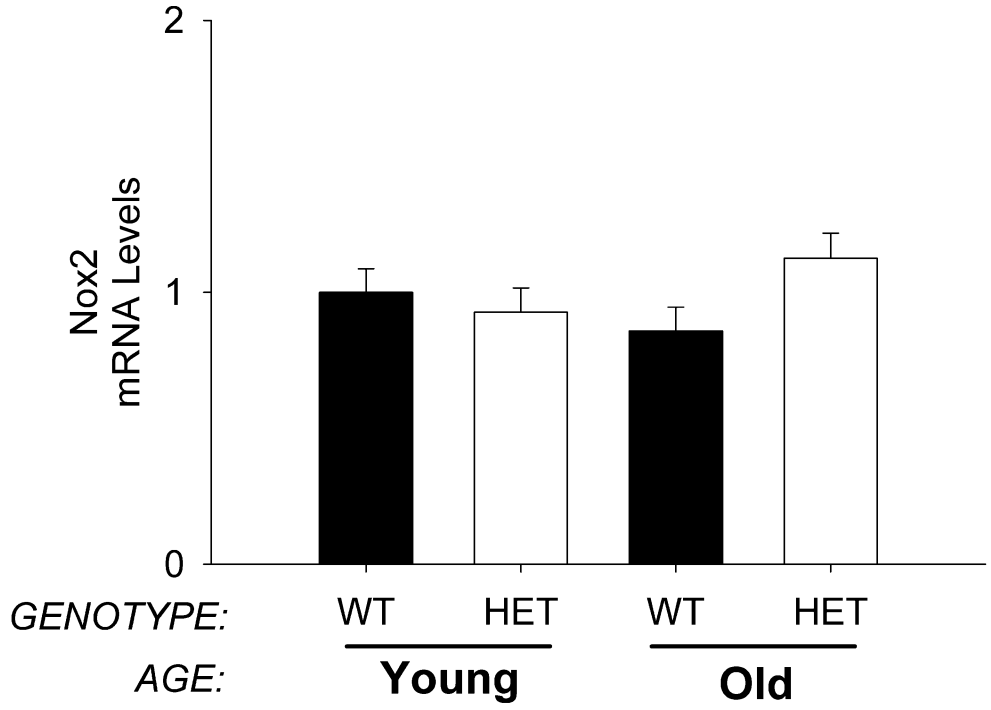

Figure 5C: ***Nox2 expression in aortic valve.***

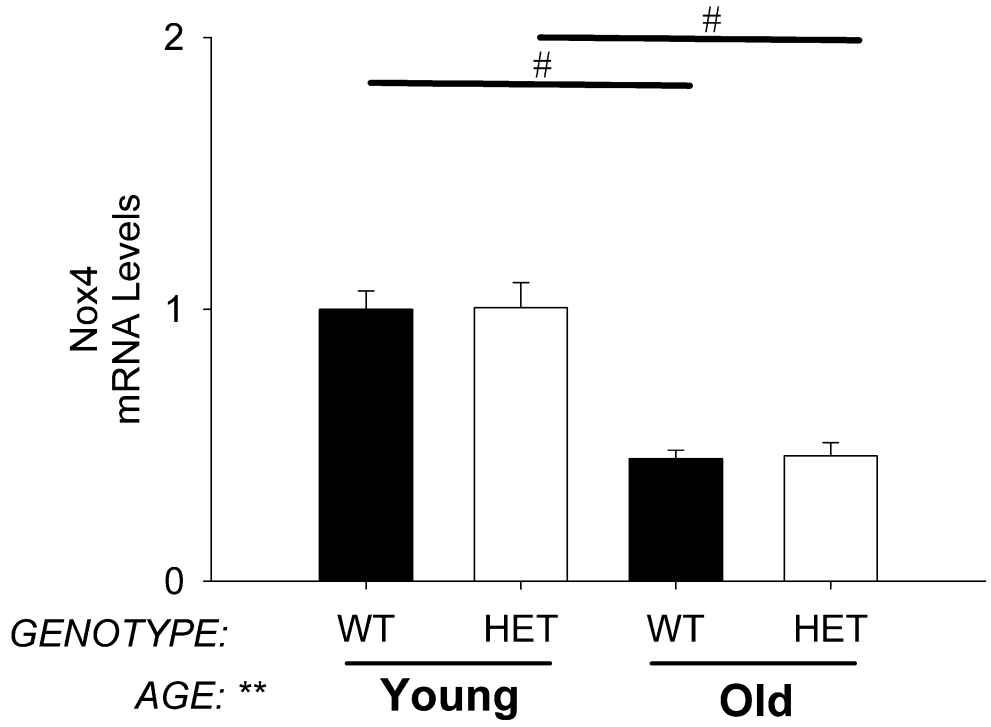

Figure 5D: ***Nox4 expression in aortic valve.***

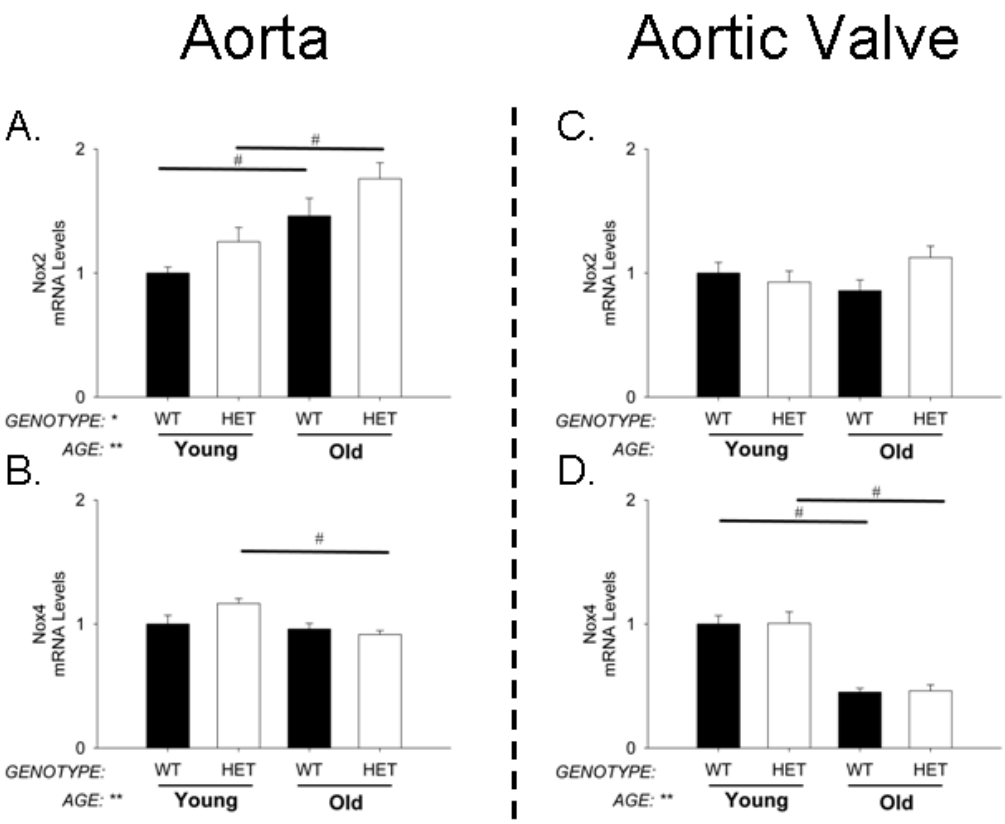

Figure 5: ***Composite***

Figure 6: *Expression of age-related genes legend.*
Note the largely unchanged Sirt expression in aorta (A-G) compared to aortic valve (I-O), whereas six of the seven Sirt isoforms were significantly reduced with age. Also, note MnSOD- haploinsufficiency largely does not impact SIRT expression. Marker of cellular senescence (Fig6H and 6O), CDK2NA, was significantly increased with age in both aorta and aortic valve. ** Denotes significant main effect of age with p-value < 0.05. # Denotes significant differences between groups with p-value<0.05. Values are means ± SE; n = 4-14 mice/group. Sirt, sirtuin.

Figure 6A: Sirt1 expression in aorta.

Figure 6B: Sirt2 expression in aorta.

Figure 6C: Sirt3 expression in aorta.

Figure 6D: Sirt4 expression in aorta.

Figure 6E: Sirt5 expression in aorta.

Figure 6F: Sirt6 expression in aorta.

Figure 6G: Sirt7 expression in aorta.

Figure 6H: CDKN2A expression in aorta.

Figure 6J: Sirt2 expression in aortic valve.

Figure 6I: Sirt1 expression in aortic valve.

Figure 6K: Sirt3 expression in aortic valve.

Figure 6L: Sirt4 expression in aortic valve.

Figure 6N: Sirt6 expression in aortic valve.

Figure 6M: Sirt5 expression in aortic valve.

Figure 6O: Sirt7 expression in aortic valve.

Figure 6P: CDKN2A expression in aortic valve.

Composite of Figure 6.

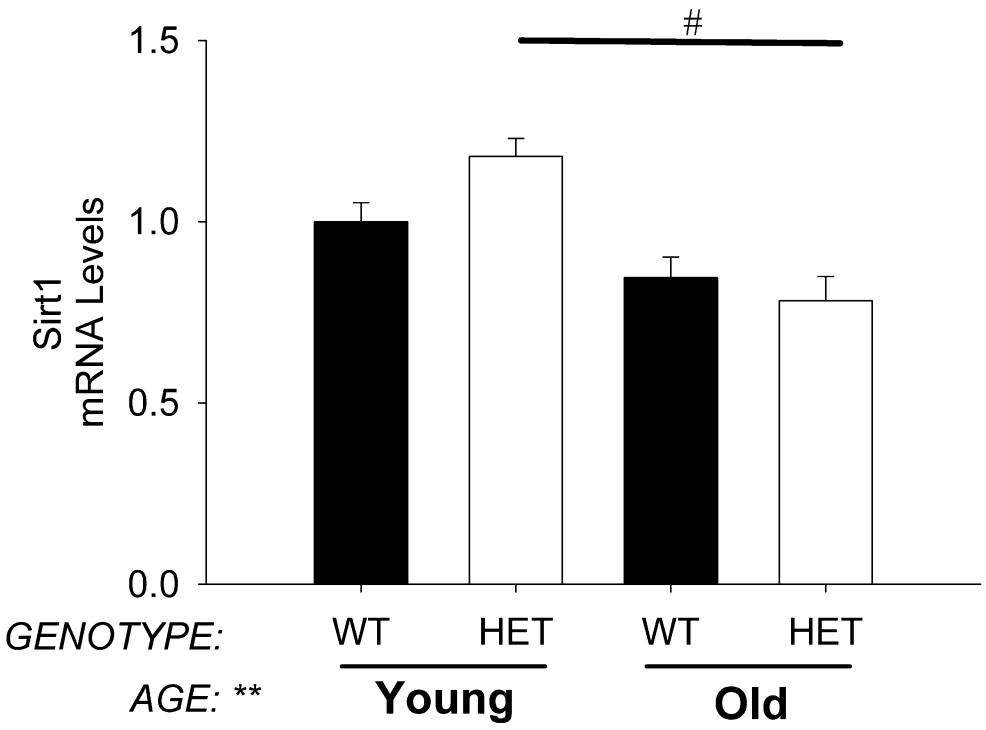

Figure 6A: ***Sirt1 expression in aorta.***

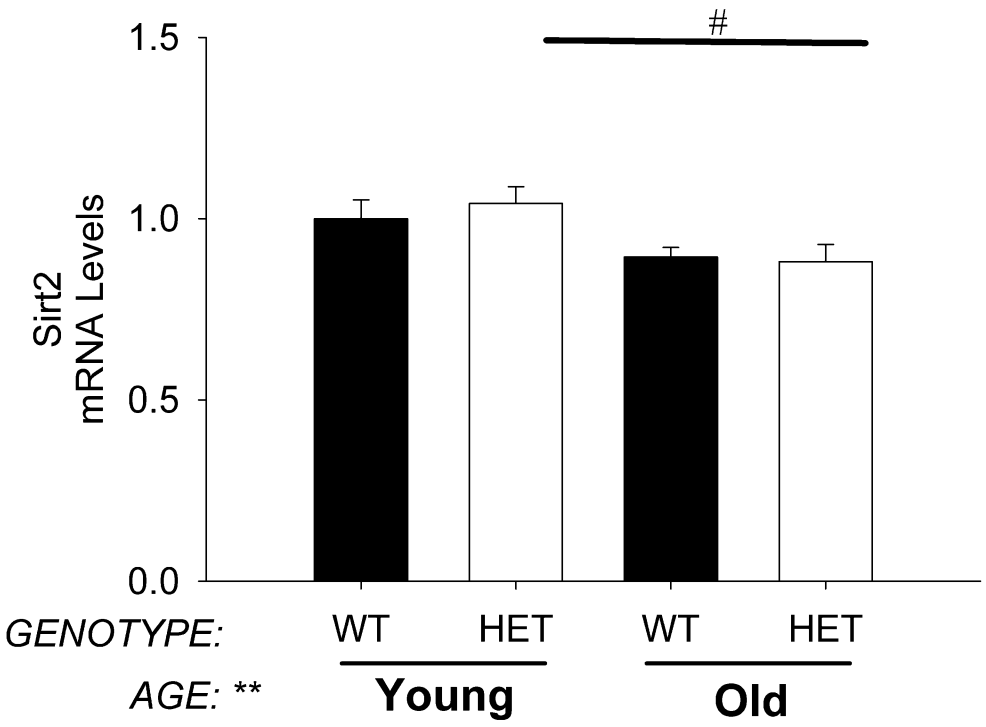

Figure 6B: ***Sirt2 expression in aorta.***

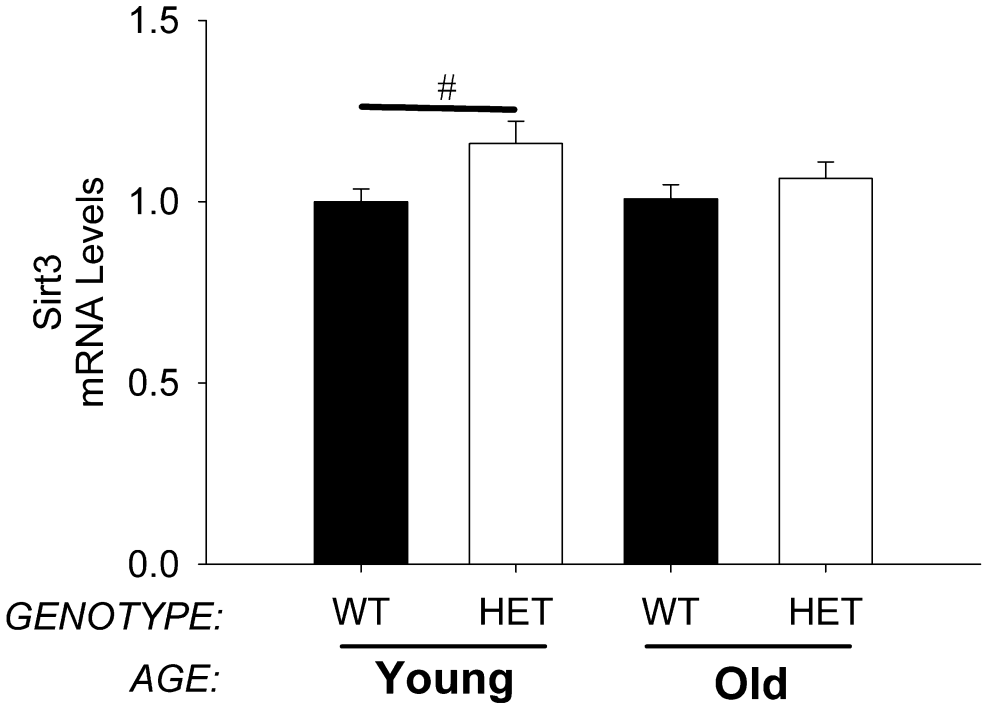

Figure 6C: ***Sirt3 expression in aorta.***

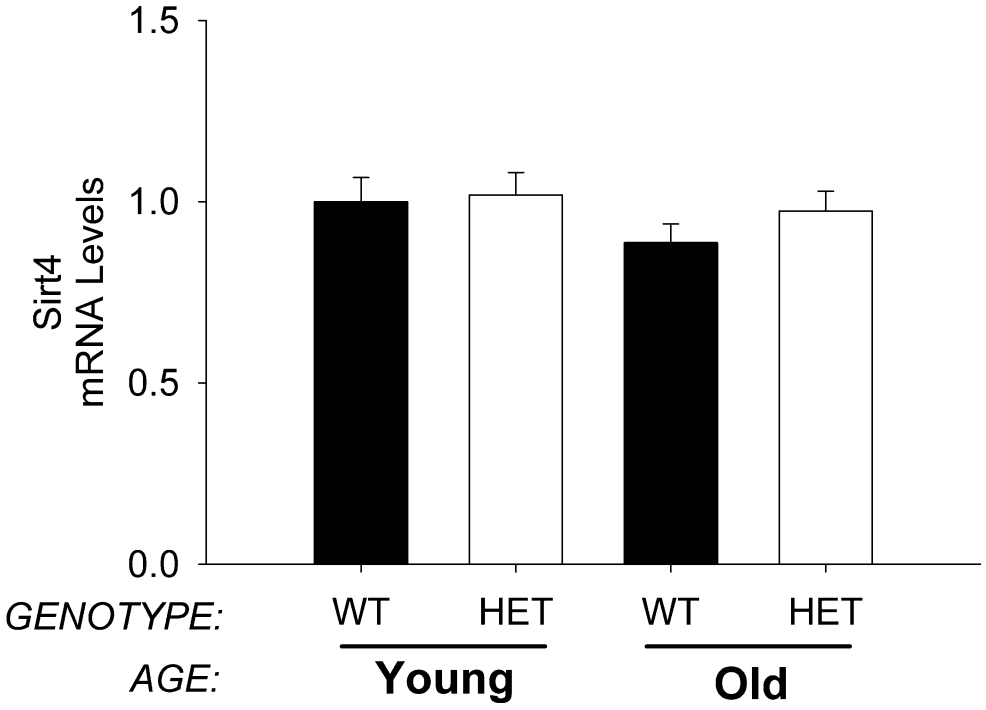

Figure 6D: ***Sirt4 expression in aorta.***

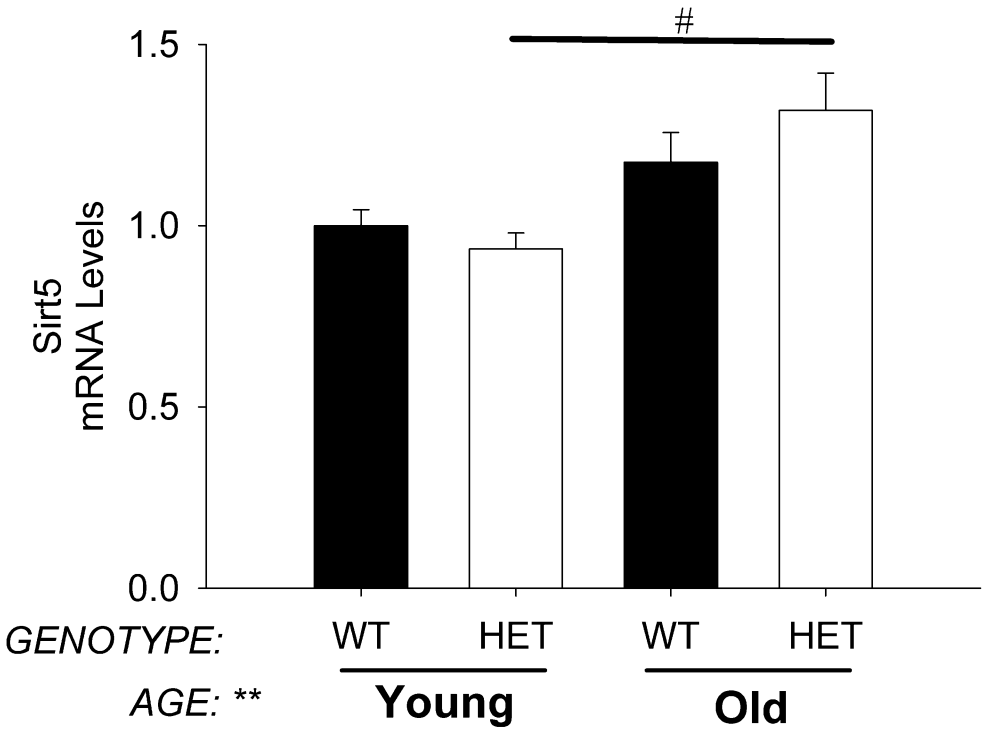

Figure 6E: ***Sirt5 expression in aorta.***

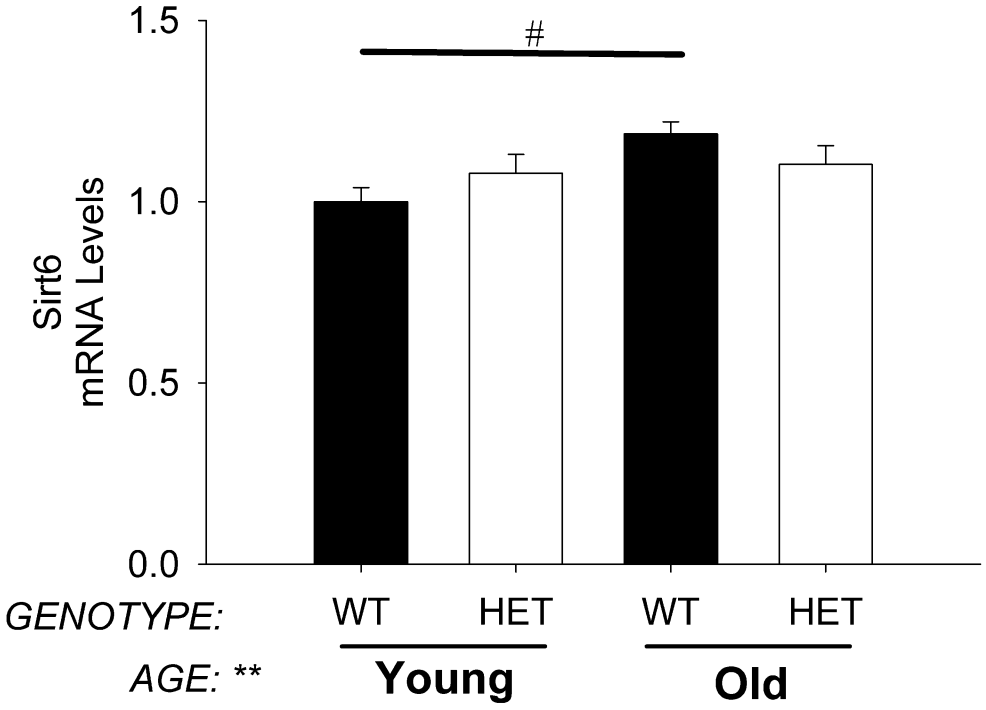

Figure 6F: ***Sirt6 expression in aorta.***

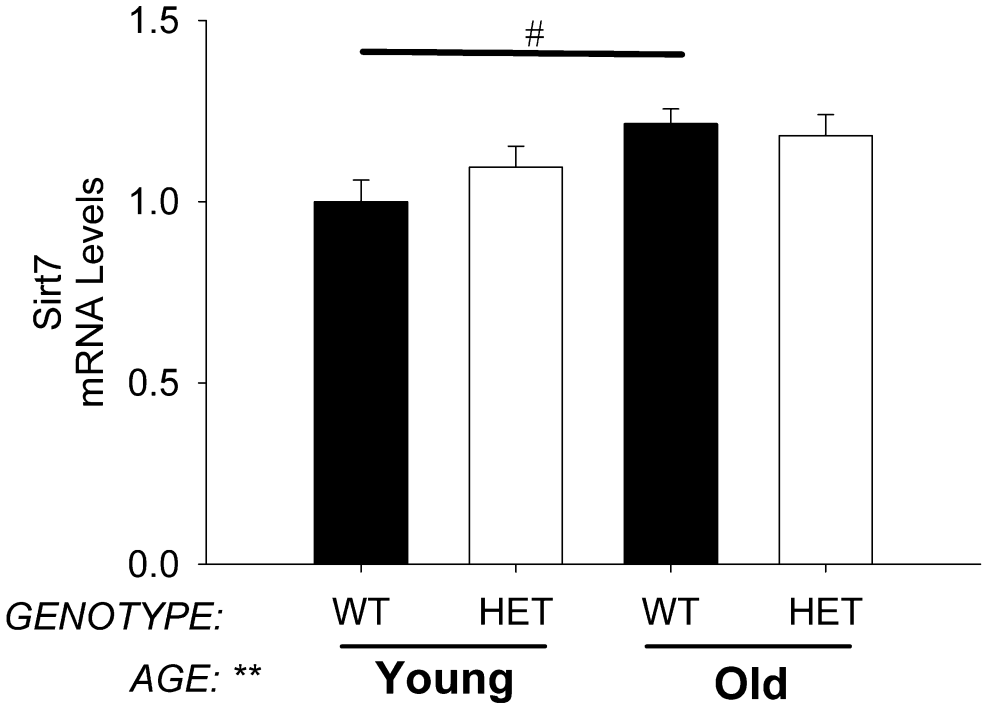

Figure 6G: ***Sirt7 expression in aorta.***

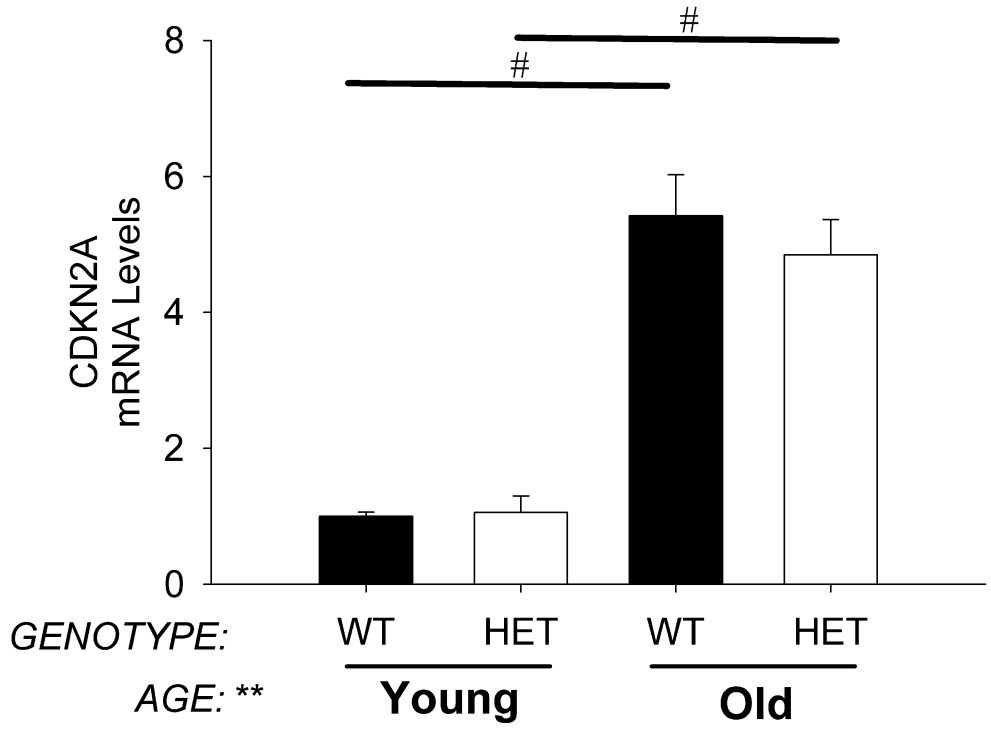

Figure 6H: ***CDKN2A expression in aorta.***

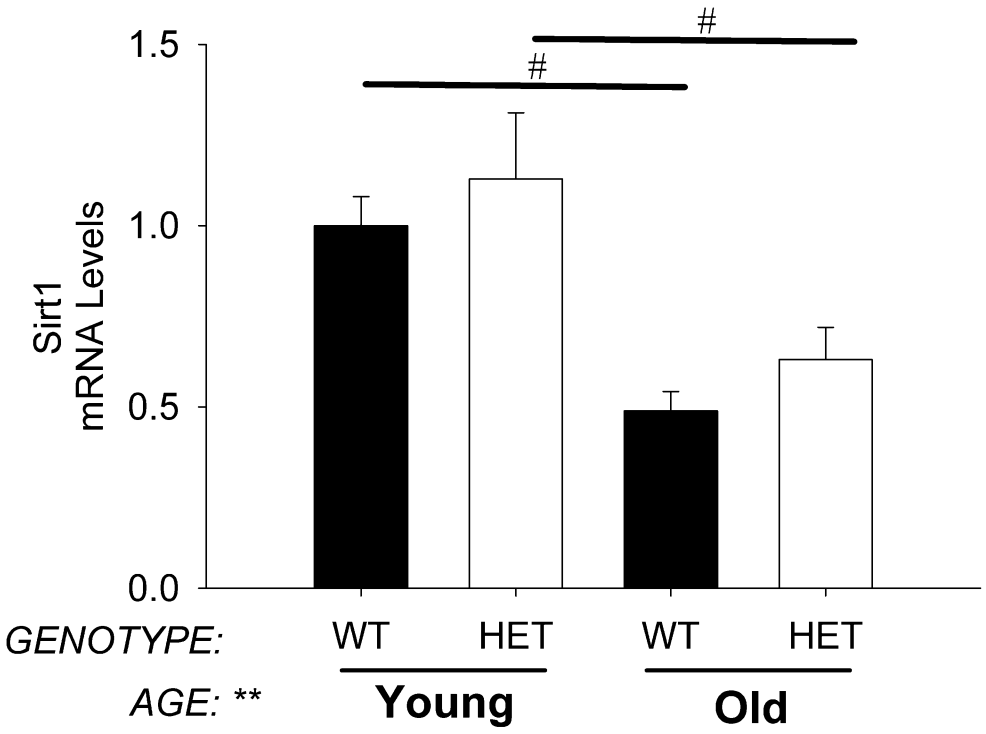

Figure 6I: ***Sirt1 expression in aortic valve.***

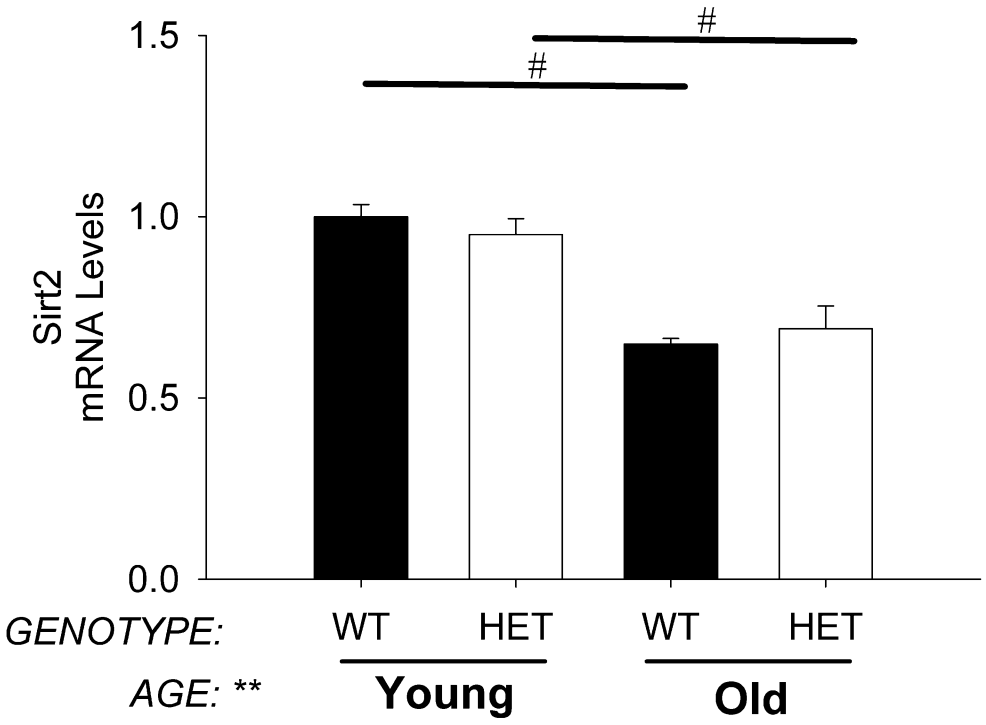

Figure 6J: ***Sirt2 expression in aortic valve.***

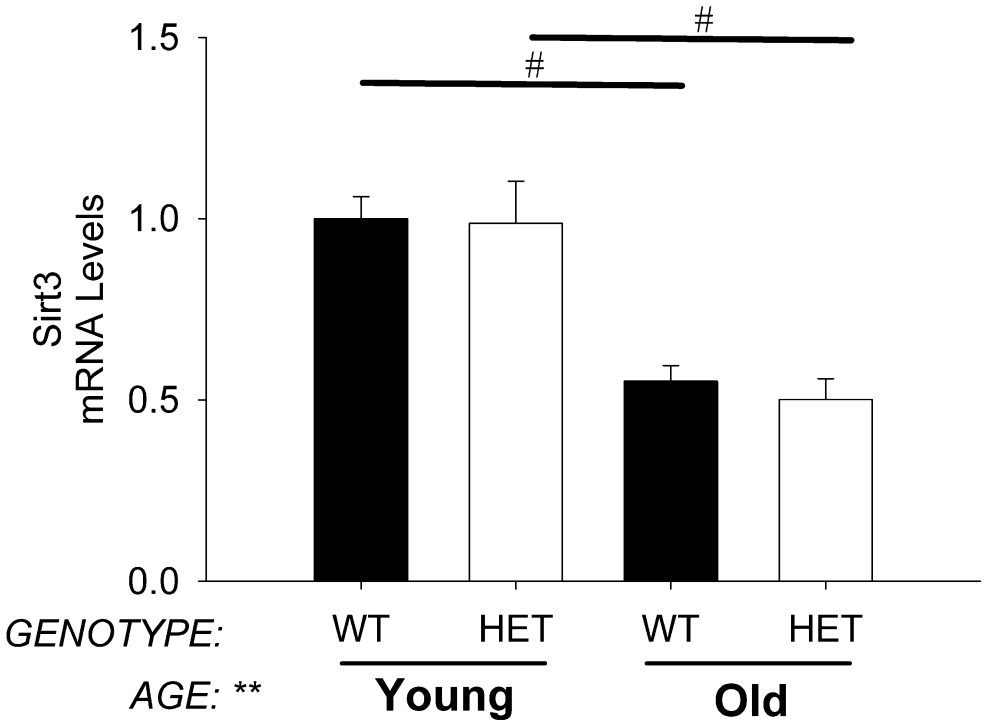

Figure 6K: ***Sirt3 expression in aortic valve.***

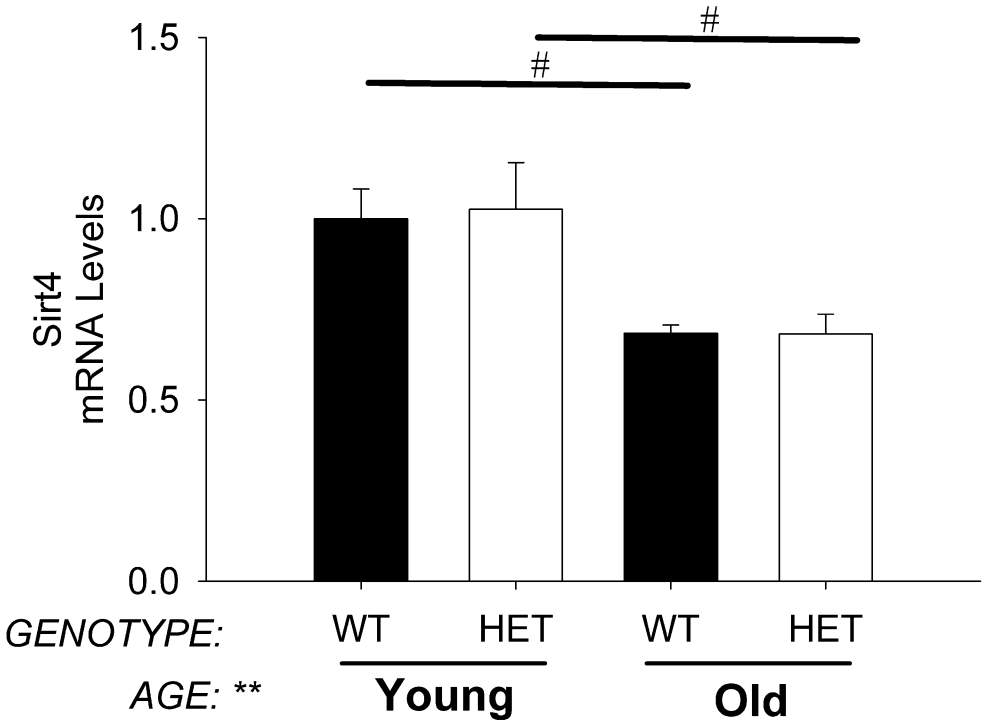

Figure 6L: ***Sirt4 expression in aortic valve.***

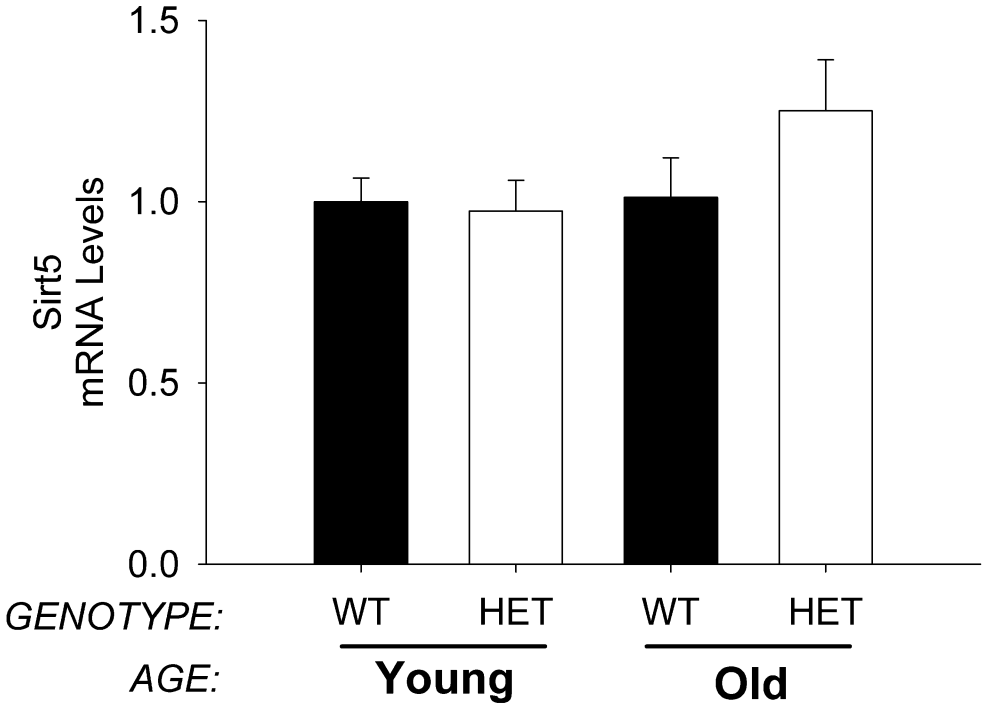

Figure 6M: ***Sirt5 expression in aortic valve.***

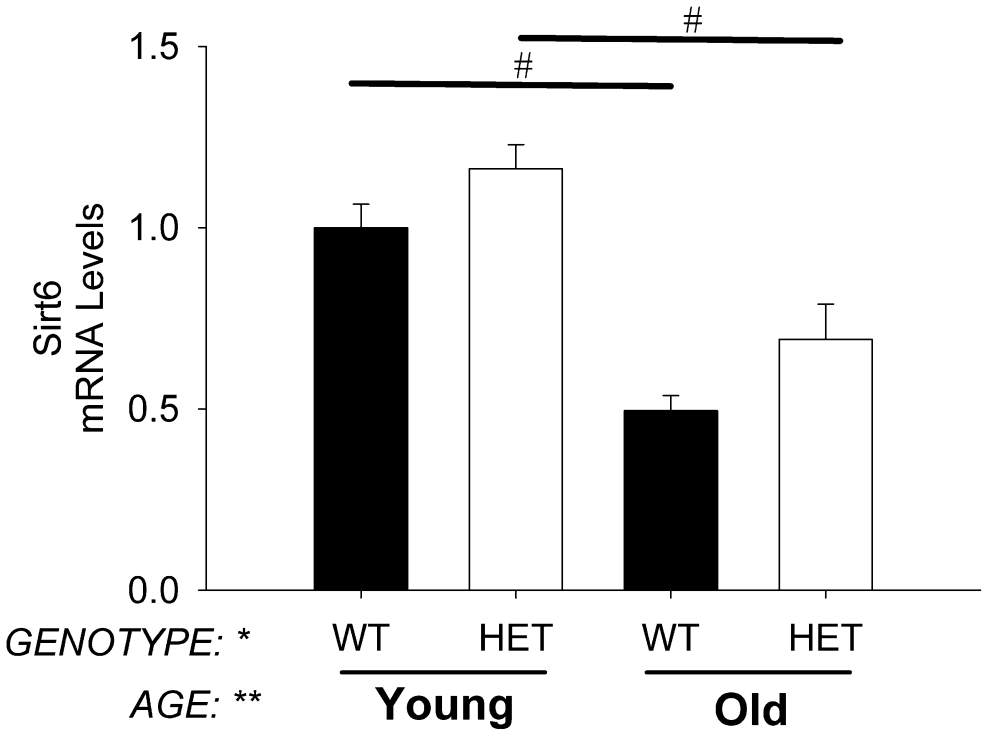

Figure 6N: ***Sirt6 expression in aortic valve.***

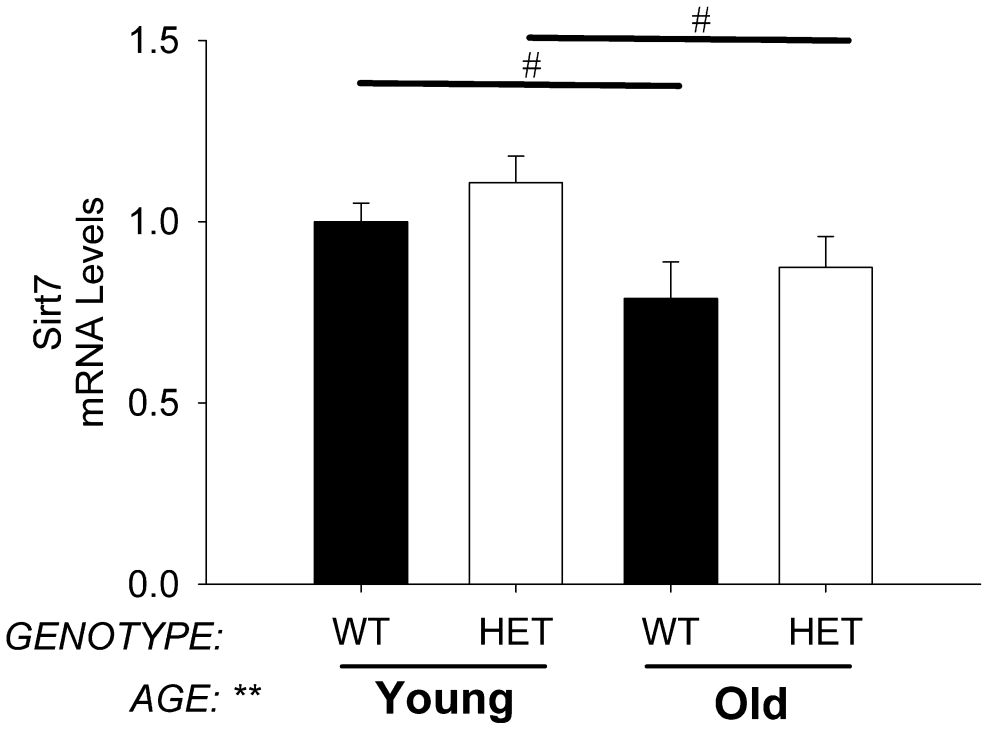

Figure 6O: ***Sirt7 expression in aortic valve.***

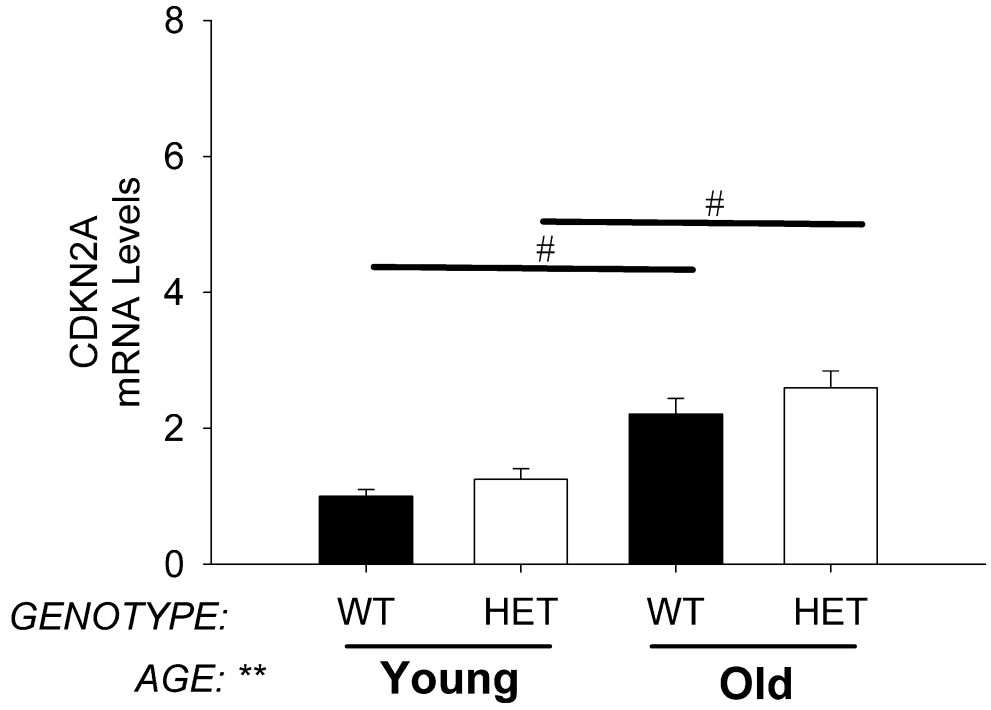

Figure 6P: ***CDKN2A expression in aortic valve.***

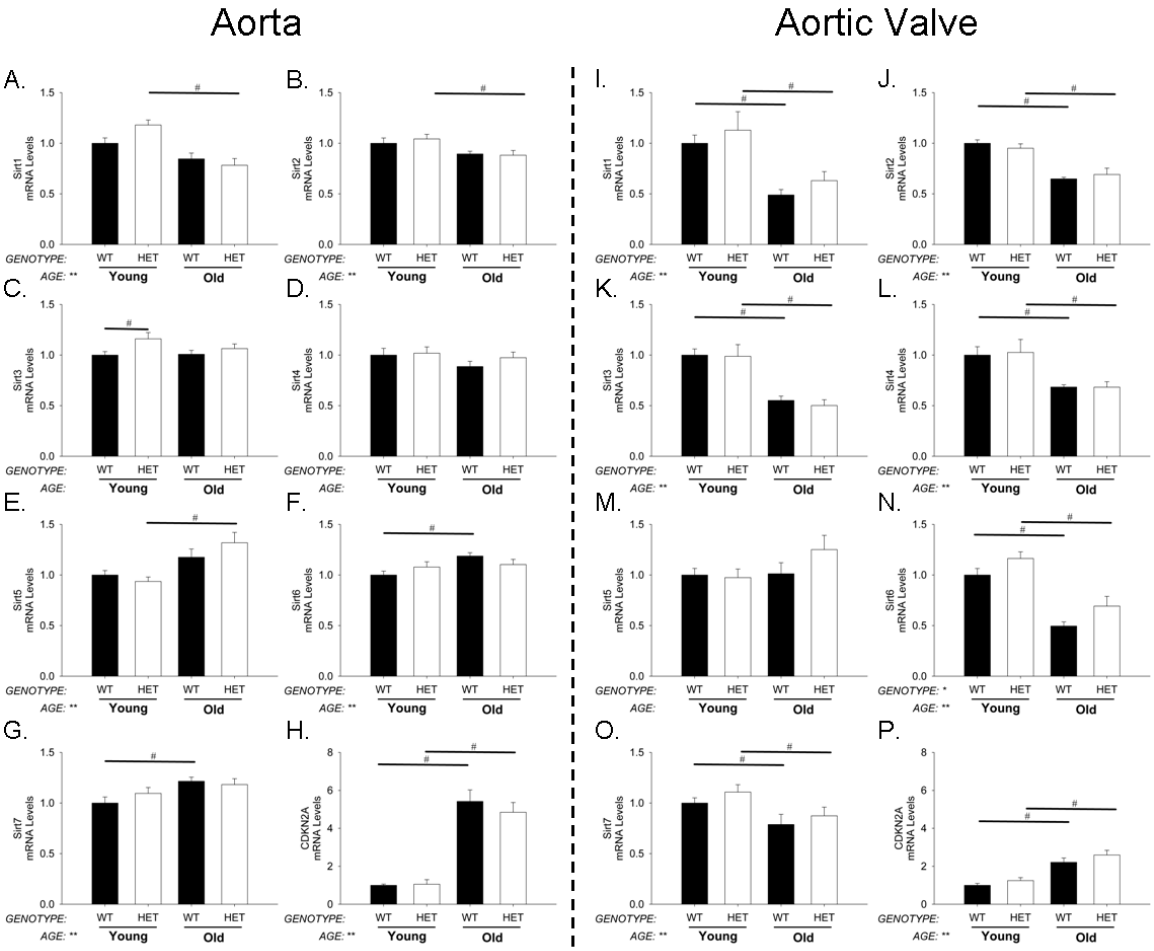

Figure 6: ***Composite***

Figure 7: ***Aorta and Aortic Valve Function legend.***
(A-B) Vasomotor responses to acetylcholine (ACh) in both young and old mice. Vessels were treated with vehicle or with apocynin, NAD(P)H oxidase inhibitor. Note the relaxation to ACh is significantly impaired in MnSOD+/- mice after incubation of apocynin. *Denotes significance in treatment group vs. control group. p-value < 0.05. (C-D) Measurements of aortic valve function by echocardiography. Values are means ± SE; n = 8-23 mice/group. Ach, acetylcholine.

Figure 7A: Aorta vasomotor function in young mice.

Figure 7B: Aorta vasomotor function in old mice.

Figure 7C: Aortic valve function measurement of cusp separation.

Figure 7D: Aortic valve function measurement of peak velocity.

Composite of Figure 7

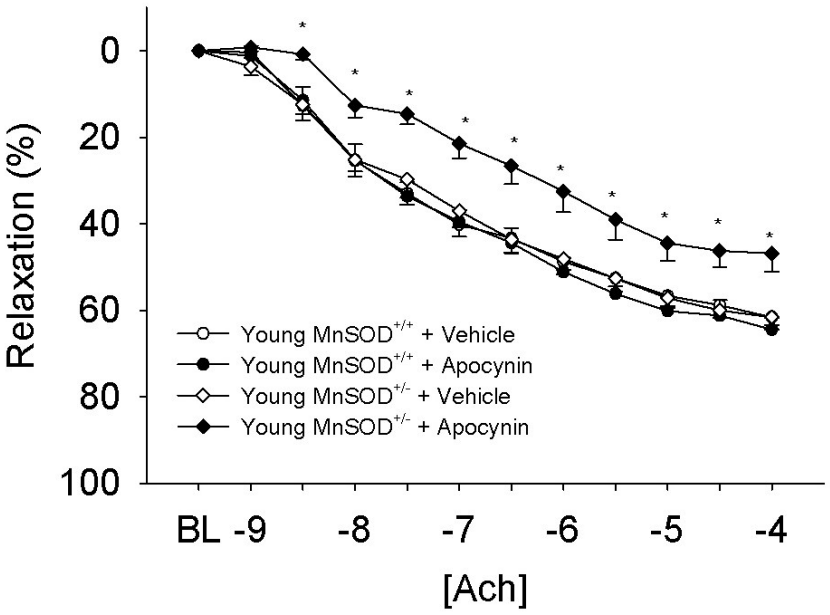

Figure 7A: ***Aorta vasomotor function in young mice.***

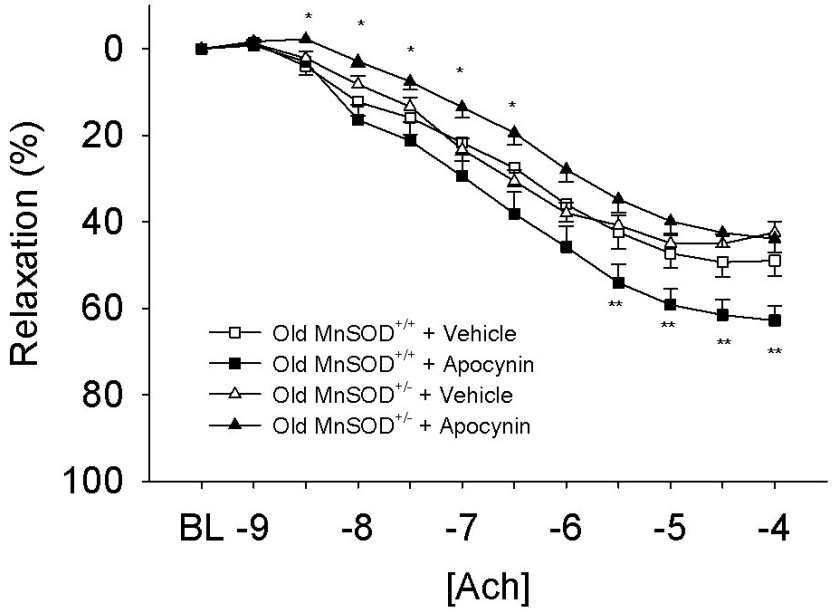

Figure 7B: ***Aortic vasomotor function in old mice.***

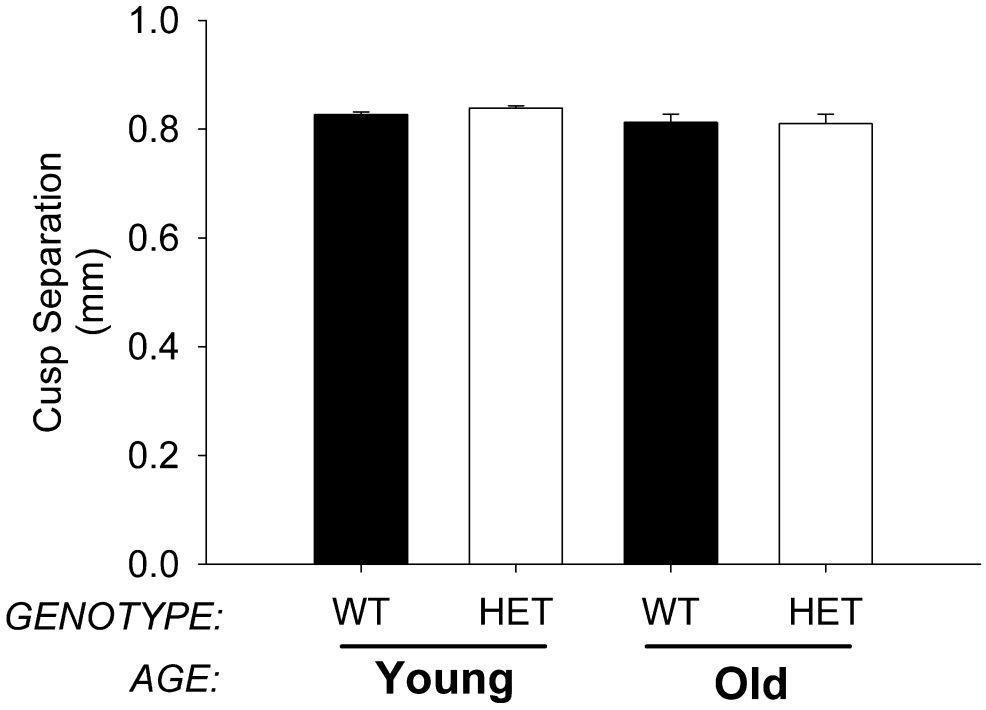

Figure 7C: ***Aortic valve function measurement of cusp separation.***

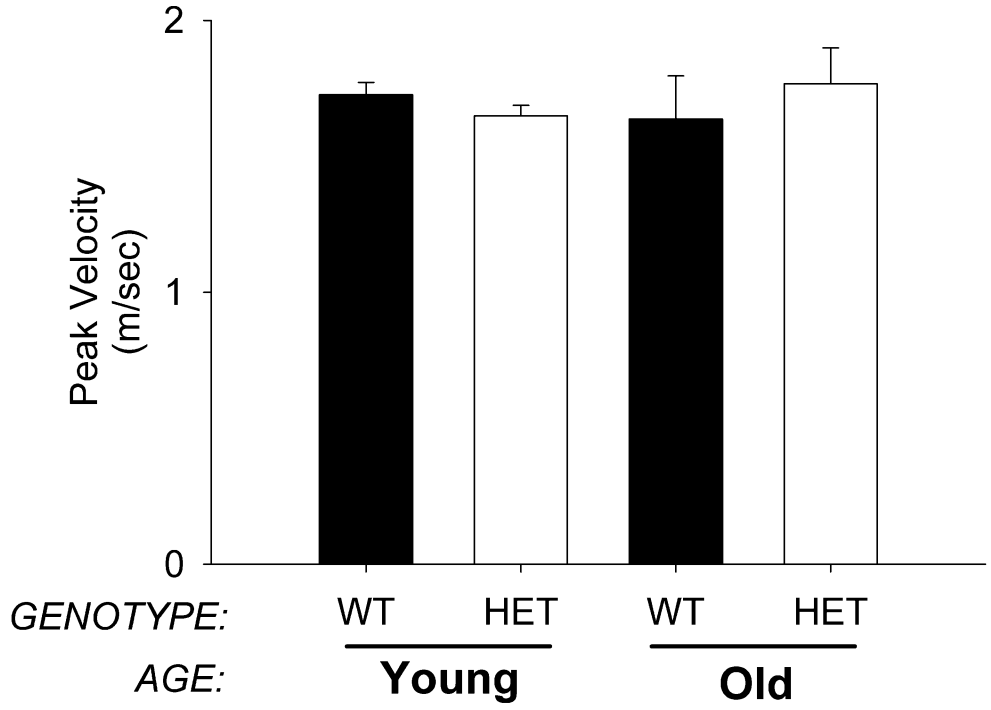

Figure 7D: ***Aortic valve function measurement of peak velocity.***

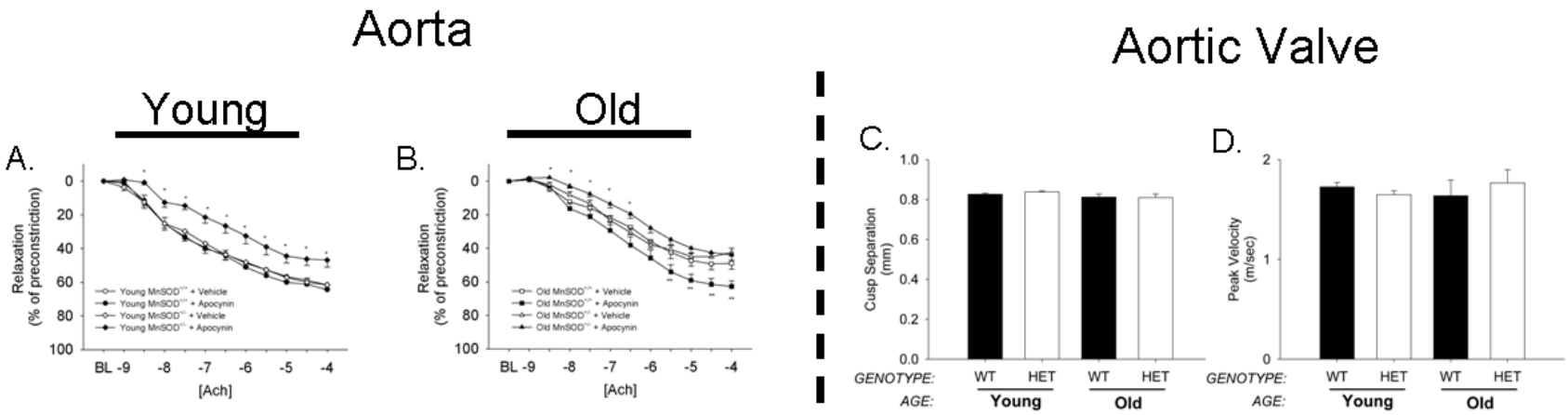

Figure7: ***Composite***

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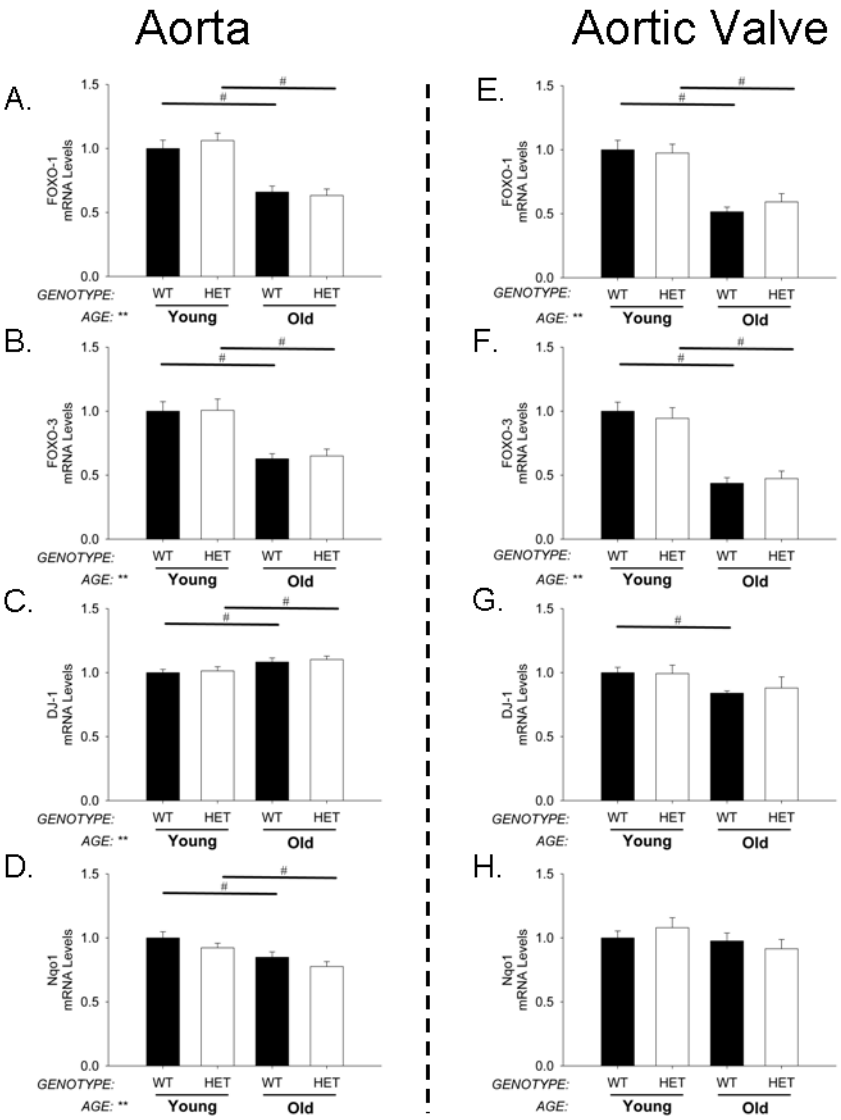
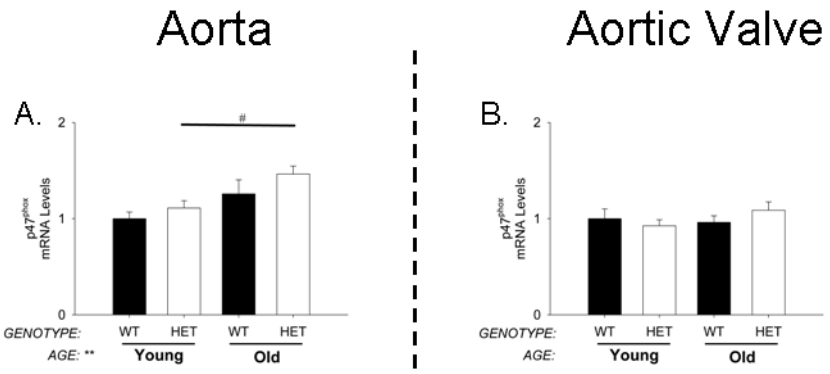
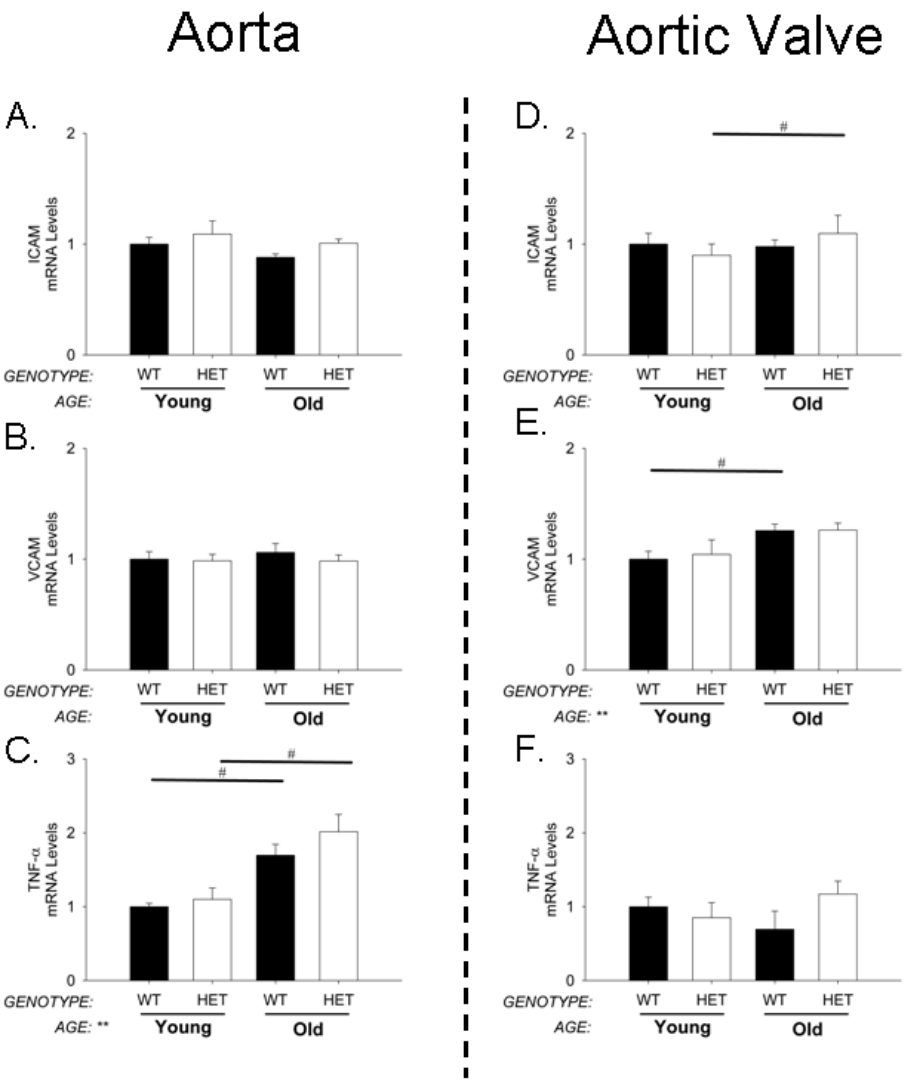
APPENDIX



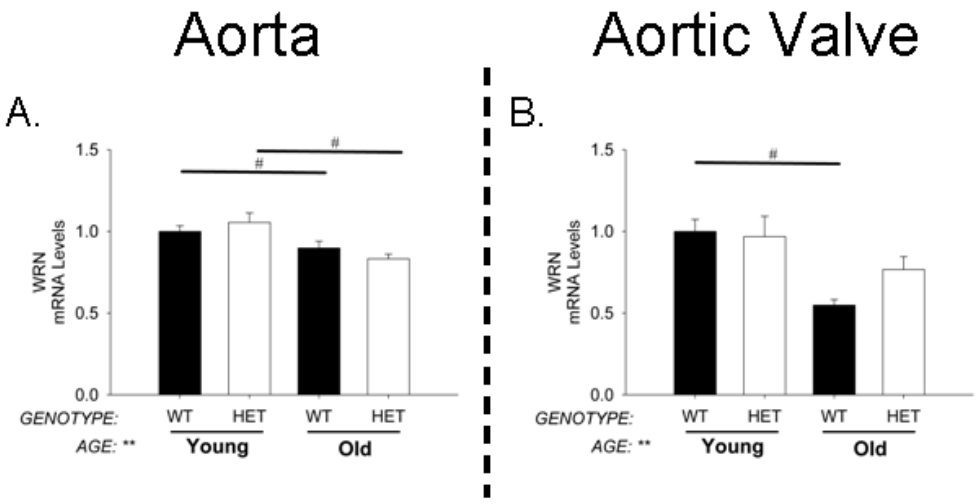
Appendix 1: ***Gene expression of genes regulating antioxidants.***
** Denotes significant main effect of age with p-value < 0.05. # Denotes significant differences between groups with p-value<0.05. Values are means ± SE; n = 4-14 mice/group



Appendix 2: *p47^{phox} gene expression of aorta and aortic valve.*
** Denotes significant main effect of age with p-value < 0.05. # Denotes significant differences between groups with p-value<0.05. Values are means ± SE; n = 4-14 mice/group.

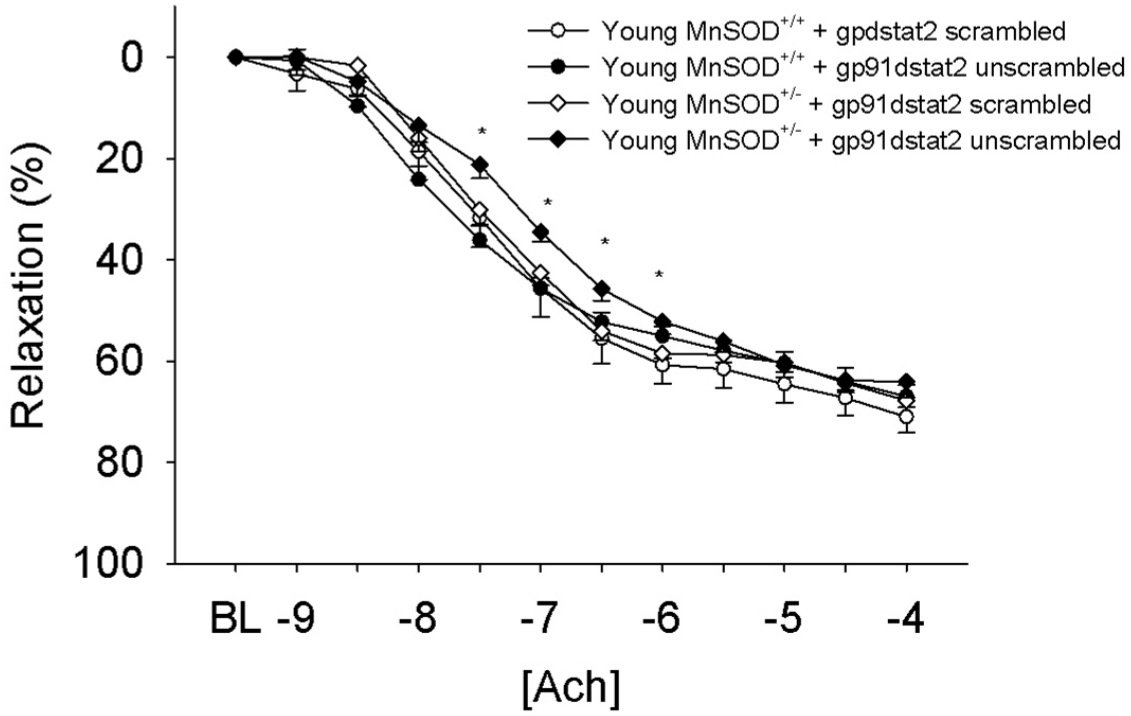


Appendix 3: ***Pro-inflammatory gene expression of aorta and aortic valve.***
** Denotes significant main effect of age with p-value < 0.05. # Denotes significant differences between groups with p-value<0.05. Values are means ± SE; n = 4-14 mice/group.



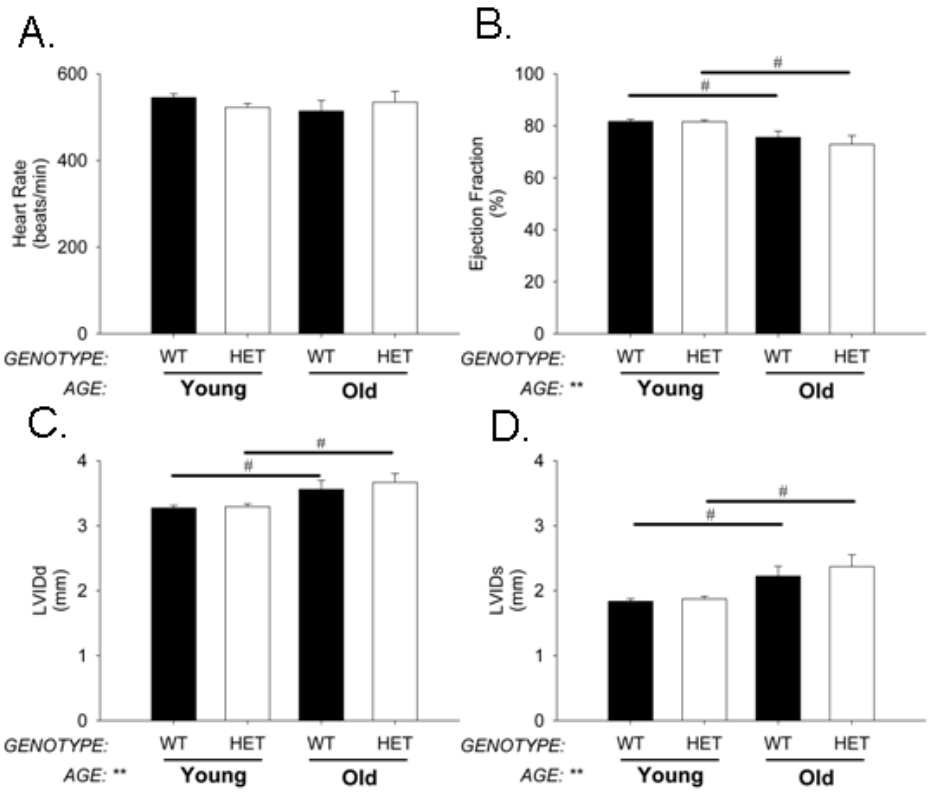
Appendix 4: ***Werner protein gene expression levels of aorta and aortic valve.***

** Denotes significant main effect of age with p-value < 0.05. # Denotes significant differences between groups with p-value<0.05. Values are means ± SE; n = 4-14 mice/group.

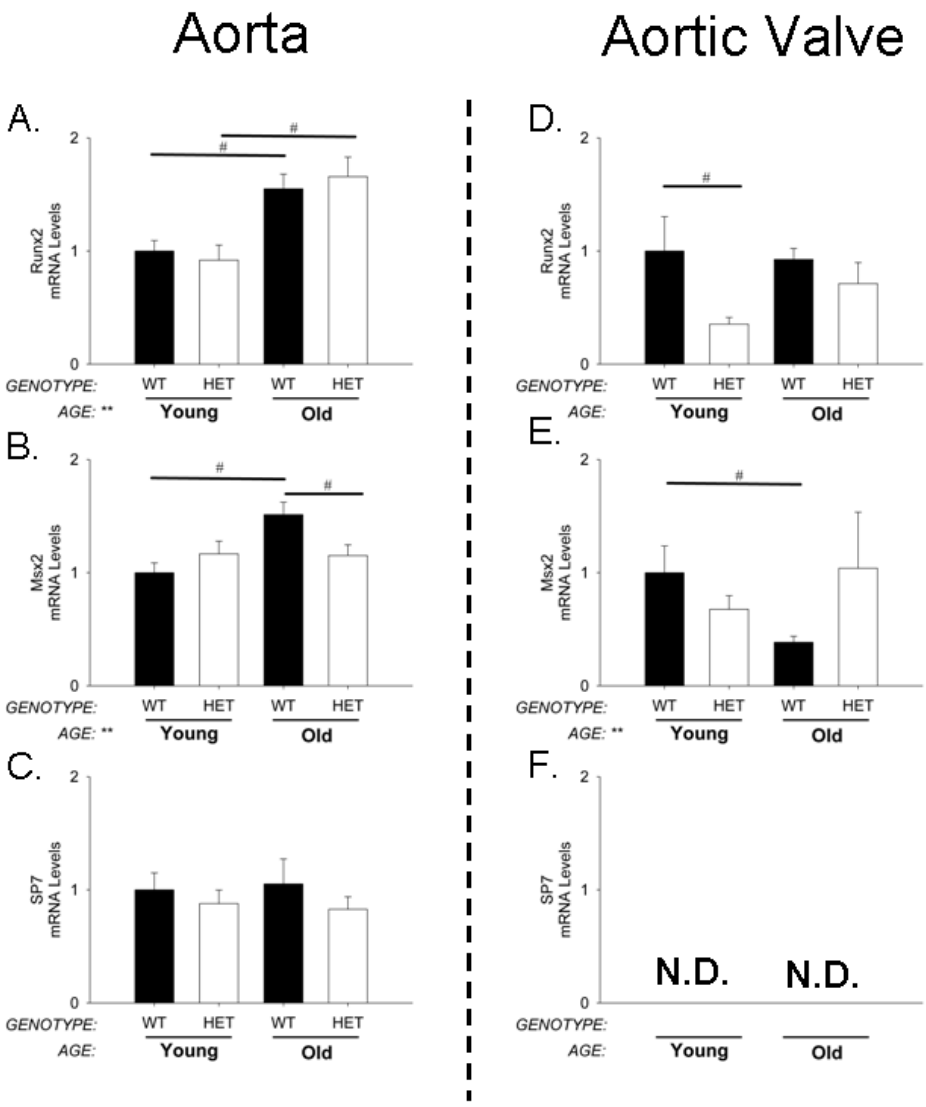


Appendix 5: ***Vasomotor responses after incubation of gp91dstat peptide 2 (500nM).***

Treatment of gp91dstat peptide 2, a specific Nox2 inhibitor, caused significant impairment of young MnSOD$^{+/-}$ mice. * Denotes significance in treatment group vs. control group. p-value < 0.05. Values are means ± SE; n = 7 mice/group.



Appendix 6: ***Cardiac Function.***
Values are means ± SE; n = 12-23 mice/group. ** Denotes significant age effect with p-value < 0.05. # Denotes significance p-value<0.05.



Appendix 7: ***Pro-calcific gene expression of aorta and aortic valve.***
** Denotes significant main effect of age with p-value < 0.05. # Denotes significant differences between groups with p-value<0.05. Values are means ± SE; n = 4-14 mice/group. N.D., not-detectable.